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STIMULATION OF MAMMARY GLANDS IN HYPOPHYSECTOMIZED RATS BY ESTROGEN AND TESTOSTERONE[1]

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IN SPITE of the generally accepted belief that estrogens and androgens fail to stimulate the mammary glands of hypophysectomized mammals, a few reports have been to the contrary. Mammary growth has been reported to occur with estrogen treatment in hypophysectomized rats (Ruinen, 1932; Freud and de Jongh, 1935), rabbits (Asdell et al., 1936) and mice (Gardner, 1940). On the other hand, Selye, Collip and Thompson (1935), Reece et al. (1936), Astwood et al. (1937), and Reece and Leonard (1941) reported no mammary growth in hypophysectomized rats treated with estrogen; McEuen et al. (1937), Noble (1939) and Leonard and Reece (1942) reported none with testosterone. Testosterone was observed to slow the rate of mammary gland involution which followed hypophysectomy in male rats, if treatment was begun immediately (Leonard and Reece, 1941).

Continued studies on this controversial question indicated that under certain specific conditions, estrogen stimulated the mammary glands of hypophysectomized rats and also that testosterone propionate modified mammary gland structure in the absence of the hypophysis. The details of these findings will be reported in this paper.

METHODS

Both the Long-Evans and Sprague-Dawley strains of rats were used in these experiments and it was found that strain differences did not affect the results. A standard adequate diet plus a .25 per cent solution of glucose was fed *ad libitum*. The age of the rats at the time of hypophysectomy varied from 22 to 36 days. Male rats were used almost exclusively.

Estradiol dipropionate[2] was injected in total amounts of 45 to 1,150 micrograms over periods of 10 to 12 days. In certain experiments, a menopause urine extract[3] was injected in total doses of 4.6 to 5.0 mg. for the same period,

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either alone or simultaneously with estrogen. Large dosages of testosterone propionate[2] were employed and no attempt was made to determine the minimal effective amounts. Treatment was begun either on the day of the operation (immediate treatment) or after an interval of 7 to 9 days (postponed treatment).

At autopsy, the right abdominal mammary glands were removed and stained whole mounts were prepared. In addition, sections of the mammary glands of the testosterone-treated rats were made. Body weights were ob-

TABLE 1. EFFECT OF ESTROGEN ON MAMMARY GROWTH IN HYPOPHYSECTOMIZED MALE RATS

EXPERIMENT	NUMBER OF RATS	AGE DAYS	BODY WEIGHT, GM.		TREATMENT		MAMMARY GROWTH
			AVERAGE	AVERAGE CHANGE	ESTROGEN μg.	MENOP. URINE, MG.	
A. Immediate treatment; estradiol dipropionate+menopause urine extract							
C1	7	27	54	+4	1150	+4.7	Positive
C5	2	28	56	−2	550	+4.9	Positive
C1ℓ	3	30	58	+3	250	+5.0	Positive
B. Immediate treatment; estradiol dipropionate							
C1	3	27	54	0	1150		Positive
C37	3	22	56	−2	50		1 Pos.; 2 Neg.
C38	3	25	64	−8	50		Positive
C47	2	25	56	−6	45		2 Pos.; 1 Neg.
C68	4	29	67	−5	50		Positive
	3♀	29	61	0	50		Positive
C. Immediate treatment; estradiol dipropionate							
C31	4	36	95	−19	440		Negative
C32	4	36	91	−19	440		Negative
D. Postponed treatment; estradiol dipropionate+menopause urine extract							
C4	6	26	66	−11	300	+4.6	Negative
C21	2	32	83	−4	500	+5.0	Negative
E. Postponed treatment; estradiol dipropionate							
C54	4	25	64	−8	45		Negative
C62	4	24	60	−6	45		Negative

served during the course of the experiments. The sellae turcicae were serially sectioned and carefully studied; data on incompletely hypophysectomized rats were not included.

RESULTS

Effect of estradiol dipropionate. In the initial experiments, menopause urine extract and estradiol dipropionate were injected simultaneously into hypophysectomized male rats, 27 to 30 days of age, and weighing on the average 54 to 58 gm. Injections were begun immediately and continued daily for 10 to 12 days. The mammary glands, when examined, were found to possess stimulated end buds characteristic of the effect of estrogen injections in normal

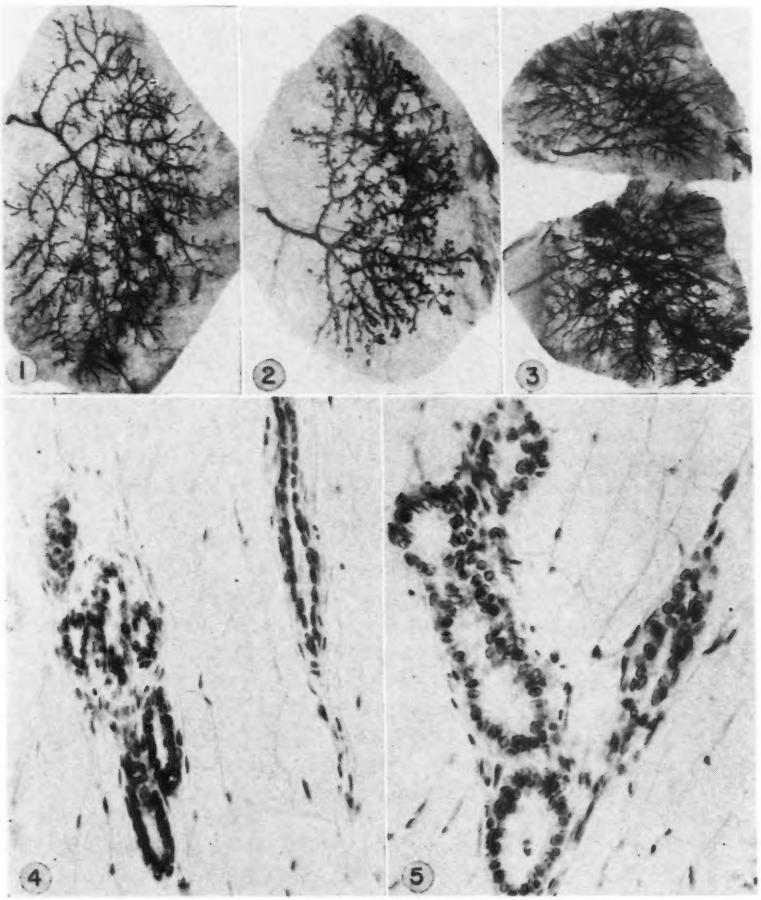

Fig. 1. (C1–2) Hypophysectomized control, 12 days after operation. Body weight, 52 gm. ×5.

Fig. 2. (C1–9) Hypophysectomized male, immediate treatment with estradiol dipropionate. Body weight, 53 gm. ×5.

Fig. 3. (C54–4) Hypophysectomized male, postponed treatment with estradiol dipropionate. Body weight, 65 gm. ×5.

Fig. 4. (C66–8) Mammary ducts (12 micra) of a hypophysectomized male rat 10 days after operation. ×675.

Fig. 5. (C66–11) Mammary ducts of a hypophysectomized male which received testosterone propionate for 10 days, immediate treatment. ×675.

or partially hypophysectomized male rats (table 1, A). Since this growth stimulation of the mammary glands in hypophysectomized rats was not obtained in previous experiments, it appeared at first sight that the gonadotropic hormone was supplying some pituitary-like mammogenic factor.

In order to determine if the extract of menopause urine acted synergistically with estradiol dipropionate to give end-bud stimulation, groups of similar

rats were treated with estrogen alone. Immature males and females 22 to 29 days of age, averaging in body weight from 54 to 67 gm. were hypophysectomized and were treated with the same or smaller amounts of estradiol dipropionate for the same period. The mammary glands of 15 of 18 rats showed similar end-bud stimulation as previously obtained with the combined hormones (table 1, B). The number of end buds stimulated and the degree of extension of the duct system were never as great as that observed with similar treatment in normal or partially hypophysectomized rats (fig. 1 and 2). Treatment with extract of menopause urine alone (6 hypophysectomized males) did not stimulate end-bud growth although the ducts appeared slightly thicker than those of the hypophysectomized uninjected controls.

Experiments were then made to determine if estrogen, with or without the extract of menopause urine, would stimulate end-bud growth after postponed treatment. Young rats within the same limits of age and body weight as above were hypophysectomized and after an interval of 7 to 8 days were injected for a 10-day period with these hormones. In no instance was any stimulation of end buds of the mammary glands observed (table 1, D and E; fig. 3). These experiments confirmed previous results in the failure to obtain mammary growth with estrogen following postponed treatment.

Examination of the data of previous investigations on this problem revealed that usually the rats were older and heavier at the time of hypophysectomy and, invariably, no mammary growth resulted from estrogen injections even when treatment was begun immediately. To determine if age and weight were factors to be considered, male rats, 36 days of age, averaging in body weight from 91 to 95 gm. were hypophysectomized and treated immediately with estrogen. At autopsy 10 days later, no evidence of end-bud growth was obtained, thus confirming the findings of previous investigators. Estrogen stimulation of the mammary glands of hypophysectomized rats occurred only when the rats were immature and weighed less than 70 gm. and the treatment was started immediately.

Effect of testosterone propionate. An investigation of the effects of testosterone propionate on mammary growth was undertaken in view of the results just presented. Litters of rats of similar ages and average body weights were employed. In addition to the hypophysectomized controls, several rats were autopsied at the time of the operation to serve as reference controls. The results obtained in the rats treated immediately or receiving postponed treatment were essentially the same and will be considered together.

Examination of the whole mounts of the mammary glands of the hypophysectomized rats receiving testosterone propionate revealed that the most outstanding effect in all cases was a thickening of the mammary ducts (table 2). There were no dilated cell-packed end buds so characteristic of estrogen-treated normal immature or the hypophysectomized immature males. The ducts forming the terminal twigs of the mammary tree were swollen so that the terminal ending of each was not distinctly marked off from the remainder of the duct. The mammary glands of the injected rats appeared to have more branches than those of the controls, but this may have been due, in part, to

the general increased thickenings of the short branches which would have made them appear more numerous. The ducts of the injected rats were definitely thicker than those of the reference controls. The hypophysectomized rats in which treatment was postponed differed from those receiving immediate treatment only in the amount of stimulation obtained. The former group showed a less extensive response than did the latter.

The extension in area of each mammary gland in the hypophysectomized rats did not exceed that in the reference controls or the hypophysectomized controls and in most cases was slightly less. This point was difficult to deter-

TABLE 2. EFFECT OF TESTOSTERONE ON MAMMMARY GLANDS OF HYPOPHYSECTOMIZED MALE RATS

EXPERIMENT	NUMBER OF RATS		AGE, DAYS	BODY WEIGHT, GM.			ANDROGEN, MG.	MAMMARY GLANDS
	EXPER.	CONTROLS		AVER.	AVER. CHANGE EXPER.	AVER. CHANGE CONTROL		
A. Immediate treatment, testosterone propionate, 10 daily injections								
C23	4	2	34	96	−4	−11	12.5	Thickened ducts
C30	4	3	35	95	−2	−7	12.5	Thickened ducts
C39	5	5	24	65	+6	+1	11.25	Thickened ducts
C46	3	1	26	71	0	−14	.045	Slightly thickened ducts
C66	4	1	25	55	+5	−4	12.5	Thickened ducts
	2	2	22	50	+4	−2	12.5	Thickened ducts
C77	3	4	23	58	+4	−5	6.9	Thickened ducts
B. Postponed treatment, testosterone propionate, 10 daily injections								
C61	2	2	25	68	+1	−2	10.0	Slightly thickened ducts
C78	3	2	24	57	−2	−5	12.5	Thickened ducts
C79	3	1	30	84	+5	+6	20.0 (16 days)	Thickened ducts

mine because variation in stretching of the glands during their preparation gave misleading conclusions unless the differences were quite large.

The same effects were obtained, regardless of the dosages of male hormone employed, but at the low levels (table 2, C 46), the amount of duct thickening was extremely small. The age and weight variations of the rats likewise made no difference in the general observations. The younger rats with the smaller initial mammary tree seemed to show a proportionally greater thickening of the ducts.

The cause of this increased diameter of the ducts was revealed by examining transverse sections of the glands and by examining the ducts of the whole mounts under high magnification of the microscope. Where the mammary gland preparations were not too thick, or too heavily stained, mitotic figure were observed in the gland cells, whereas they were never encountered in the cells of the control glands. Another cause for the thickening of the ducts was seen in the increase in size of the individual cells (fig. 4 and 5). The nuclei of the cells of the ducts in the hypophysectomized control rats were irregular in shape and chromophilic. The amount of cytoplasm about each cell was small and the epithelium of the ducts appeared to be a low cuboidal or a slightly

flattened cell type. Under the influence of the male hormone the nuclei were swollen and rounded, with either smaller amounts of chromatin material, or else it was more widely dispersed within the enlarged nuclei. The nuclei appeared to stain lighter. The most striking change was the increase in the amount of cytoplasm in each cell so that the epithelium of the ducts was entirely cuboidal or in many places a low columnar type. This increase in the amount of cytoplasm made the nuclei appear more widely separated.

The swollen ducts had widely dilated lumina in contrast to those of the controls. Secretory material was seen in the lumina of the stimulated ducts which appeared to be derived from the cytoplasm of the lining cells. In some places, portions of the cytoplasm of the cells appeared to be forming this secretory material in a manner not unlike that reported for the formation of milk. In contrast, the lumina of the ducts of the control glands were either absent or very small, and no secretory material was present in them.

DISCUSSION

It was found that if immature rats between the ages of 22 to 30 days, weighing not over 70 gm., were hypophysectomized and were injected immediately with adequate doses of estradiol dipropionate for a period of 10 to 12 days, their mammary glands possessed stimulated end buds. The degree of stimulation of the mammary glands of the hypophysectomized rats was small when compared to the results obtained with normal or partially hypophysectomized rats, yet the significance of these results is increased since no growth was obtained when these conditions were not fulfilled. No end-bud stimulation was obtained when the rats were older and heavier at the time of hypophysectomy or if the injections were delayed after the operation, regardless of the age and weight of the rats.

These results do not conflict with previous findings because in most instances, the conditions of other experiments reported were not the same (Reece, 1936; Selye et al., 1935; Astwood et al., 1937; Reece and Leonard, 1941). One notable exception was that reported by Nathanson, et al. (1939) in which rats weighing 50 to 70 gm. were hypophysectomized and injected immediately with estrogen yet no mammary stimulation was obtained. Since the period of treatment was extended from 3 to 5 weeks before autopsy, a regression of the end buds might have occurred.

It does not seem probable that residual pituitary mammogen in the hypophysectomized rats reacted synergistically with the estrogen to give end-bud growth (Reece and Leonard, 1941) because this would be equally true for older rats in which no growth was obtained. At first sight, there appeared to be a relationship between body weight changes after hypophysectomy and mammary gland responses to estrogen. Young hypophysectomized rats normally continue to gain weight slowly after hypophysectomy and it was in such rats that mammary growth was obtained. However, in some instances losses in body weight were as great in the rats receiving immediate treatment as in those in which treatment was postponed, yet the former group alone showed end-bud growth.

The influence of age and body size on the growth response of the mammary glands of the hypophysectomized rats suggests that the component tissues possess certain potentialities for hyperplasia which can be activated by estrogens during certain periods of immaturity. That this ability to respond to estrogens is subsequently lost was indicated by the absence of mammary growth in the larger and older animals. Gardner in 1940 reported that estrogens stimulate mammary growth in hypophysectomized mice of various ages as well as at a variety of intervals subsequent to the operation. This may indicate that the mammary tissues of the mouse retain for a considerable time this ability to undergo hyperplasia when stimulated by estrogens, even in the absence of the hypophysis. Several strains of mice show a high susceptibility to the spontaneous occurrence of mammary tumors (Gardner, 1941). In many instances, estrogens increase the tumor incidence and cause an appearance of the tumors earlier than usual. Species differences and differences among strains of animals may account for the variations in mammary growth responses of hypophysectomized animals to estrogen.

The results of testosterone propionate injections showed quite clearly that age and body weights of the hypophysectomized rats, or the time at which treatment was started, played no part in the essential characteristic changes in the mammary glands. The ducts throughout were thickened and dilated. This was brought about by an increase in the size of the lumina, a hyperplasia of the epithelial cells, and a hypertrophy of the epithelial cells involving both the cytoplasm and the nuclei.

These specific changes in the mammary duct system may be considered as 'growth phenomena' in the broad meaning of the term. Rats, in which the pituitary is intact or to which adequate pituitary hormones are supplied, respond to male hormone treatment by a development of the lobular-alveolar components of the mammary gland (Selye et al., 1936; Nelson and Gallagher, 1936; Reece and Mixner, 1939; Reece and Leonard, 1942). The previous statements, that male hormone does not stimulate growth of the mammary glands in hypophysectomized rats (McEuen et al., 1937; Noble, 1939; Leonard and Reece, 1942), may have been the result of the observers stressing the failure of lobular-alveolar growth to appear in these hypophysectomized animals. In the present work male hormone injected into hypophysectomized rats stimulated all the structures that it did in normal rats with the exception of the formation of the lobular-alveolar components. In still older male rats in which lobular formation was already present at the time of hypophysectomy, male hormone slowed the rate of involution of the lobules (Leonard and Reece, 1942). It is possible that some of these lobules also might have been stimulated as a result of the injections.

That pituitary mammogen is necessary for lobular-alveolar growth in the mammary gland of hypophysectomized castrated adult female rats has been shown (Reece and Leonard, 1942). The pituitary mammogen which acts synergistically with the sex hormones to induce mammary development similar to that obtained in normal rats is stated to be the lactogenic hormone, (Lyons et al., 1942; Gardner and White, 1941), but this has been denied by

others (Mixner et al., 1941). The results reported in this paper support evi dence on a possible mechanism of this synergistic action and also indicate that the lactogenic hormone may be the pituitary mammogen. Since male hormone has been shown to stimulate growth of the mammary glands of hypophysectomized rats, the action of the pituitary mammogen would be to augment this growth effect already under way and to carry it to complete lobular-alveolar formation. The fact that slight secretory activity was induced by male hormone alone in the hypophysectomized rat seems to indicate that the lactogenic hormone, whose function is considered primarily to induce milk secretion, might be the hormone to carry this growth effect on to completion also. That the lactogenic hormone and the sex hormones together may not be sufficient to produce a fully developed mammary gland has been suggested (Lyons et al., 1942). The addition of adrenotropic and growth hormones to the lactogenic hormone proved more efficacious in obtaining lobular-alveolar development with sex hormones in hypophysectomized rats.

SUMMARY

It was found that estradiol dipropionate stimulated growth of the mammary gland end buds in hypophysectomized immature male and female rats if *a*) the normal healthy rats weighed less than 70 grams at the time of hypophysectomy, *b*) the injections were begun immediately after the operation and *c*) the glands were examined after 10 to 12 days of treatment. No stimulation of the mammary gland end buds was obtained in hypophysectomized rats if *a*) the rats were significantly heavier than 70 grams at the time of the operation regardless of when hormone treatment was begun, *b*) a period of 7 days elapsed before treatment was begun regardless of age or weight of the rat. It is possible that the mammary gland cells retain, for a certain period of postnatal life, potentialities for growth which can be stimulated by estrogen, but after this critical period, these potentialities are lost forever and the cells are unaffected by estrogen.

Injections of testosterone propionate into young hypophysectomized male rats resulted uniformly in thickening of the ducts of the mammary tree. This effect was caused by some hyperplasia and by hypertrophy of the epithelial cells, and by an increase in the diameter of the lumen of the ducts. Secretory material was observed in many ducts of the stimulated glands and could be seen forming from the cytoplasm of the epithelial cells. This finding indicates a possible mechanism of the synergistic action of testosterone and the pituitary mammogen in which the latter substance not only augments the effects of testosterone but also permits lobular-alveolar growth to occur.

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INFLUENCE OF AGE ON THE EFFECT OF THYROIDECTOMY IN THE RHESUS MONKEY[1]

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THE EXPERIMENTS to be reported in this paper were primarily intended to supplement our knowledge of the effects of thyroid insufficiency in man. The rhesus monkey (*Macaca mulatta*) was chosen as the experimental animal since we assumed that thyroidectomy in any catarrhine primate would have the same effects as in man. Unexpectedly, however, our first experiments showed this assumption to be wrong. Total thyroidectomy had no apparent effect on the adult rhesus monkey.

A review of the older literature revealed conflicting reports concerning the effect of thyroidectomy on monkeys. Horsley in 1884 and 1886 was the first to report extirpation of the thyroid in monkeys. Besides symptoms of tetany, which would be referred today to lesions of the parathyroid, he observed gradual development of 'the symptoms of the disease termed myxedema by Dr. Ord, and ordinary cretinism.' Most of Horsley's experiments seem to have been on young monkeys (species not given), but he stated that 'an older animal, if kept under ordinary circumstances, will survive for 6 or 7 weeks, dying at the end of that time of myxedema.'

Murray, in 1893, noticed inactivity, swelling of the lips and eyelids, and anemia following thyroidectomy in a bonnet monkey (*Macaca radiata*). These symptoms were relieved by injection of thyroid extract. Edmunds (1900), who did not state the species of monkey that he used, noticed swelling of the face in 1 of 10 animals on which thyroidectomy was performed. Von Eiselsberg (1895) described an acute onset of symptoms of tetany and gradual development of sluggishness and apathy following thyroidectomy in a young Barbary ape (*Macaca sylvanus*). Furthermore, Burford, Allen and Diddle (1936) reported a marked increase of body weight and sluggishness in 4 adult female spayed rhesus monkeys after thyroidectomy.

On the other hand, Munk (1897), Kishi (1904), Vincent and Jolly (1904 and 1906), Halpenny and Gunn (1911), and Carlson and Woelfel (1910) reported a series of negative experiments. In the older work the species was not stated. Vincent and Jolly used both macaca and cercopithecus; Halpenny and

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Gunn, *Macaca mulatta*. Kishi stated that soon after operation convulsions occurred which sometimes killed the animals. The surviving animals never exhibited symptoms of myxedema or mental apathy and never any swelling of the skin. Munk (1897) made very careful autopsies and found no trace of thyroid tissue or only minute amounts in the 17 animals operated on. Carlson and Woelfel (1910) found that a rhesus monkey in whom a (presumably total) thyroidectomy had been performed, showed no evidence of even 'incipient myxedema.'

Although these reports and our first experiments showed that the effects of thyroid deficiency in man could not be duplicated in the rhesus monkey, it seemed of interest to continue the study of the effects of total removal of

TABLE 1. DATA ON THYROIDECTOMIZED MONKEYS

ANIMAL NUMBER	SEX	WEIGHT AT AT OPERATION GM.	AGE AT OPERATION, APPROXIMATELY	SURVIVAL TIME DAYS	CAUSE OF DEATH
671	F	4680	Adult	63	Tuberculosis
696	F	3560	Adult	44	Tuberculosis
697	F	3800	Adult	163	Tuberculosis
701	F	4450	Adult	487	Tuberculosis
S 5	M	3680	Adult	113	Tuberculosis
S 6	M	4080	Adult	80	No autopsy
S13	M	2850	2.5 years[1]	134	Tuberculosis
S 7	M	1090	8 months[1]	7	Post-operative
S 8	F	1140	8 months[1]	151	Anesthesia

[1] The age was estimated from the tables of Schultz, (1933).

the thyroid gland by the same methods that had proved useful in our earlier work on children (Wilkins, Fleischmann and Block, 1941).

MATERIAL

In the present paper are reported studies on 9 rhesus monkeys (*Macaca mulatta*) on which total thyroidectomy was performed (table 1).

The thyroid was removed under nembutal anesthesia. The completeness of the thyroidectomy was demonstrated both by anatomic search and by physiologic tests with thyrotropin. These studies will be reported in detail later in this paper. No effort was made to preserve the parathyroids. Soon after operation 4 animals developed convulsions which were relieved by injections of calcium gluconate and parathyroid hormone. In one animal, the calcium level of serum taken during a convulsion was 6 mg. per cent. It appears logical to assume that these convulsions were due to operative injury to or removal of parathyroid tissue.

METHODS

In the light of our earlier work with human beings we complemented our clinical observations with laboratory studies of B.M.R., level of serum cholesterol and urinary creatine excretion, all of which are influenced by thyroid

activity not only in man (Wilkins, Fleischmann and Block, 1941) but also in rabbits (Fleischmann et al., 1940). That these laboratory tests for measuring thyroid activity are as valid in the rhesus monkey as in man and rabbit is evidenced by the following experiment: A single intramuscular injection of thyroxin (1 mg.) was given to a thyroidectomized monkey and a marked change noted in B.M.R., serum cholesterol and urinary creatine. The metabolic rate rose from 44.1 to 56.8 cal./kg./24 hours, serum cholesterol dropped from 142 to 85 mg. per cent, and urinary creatine output rose from 15 to 44 mg. daily.

The B.M.R. was studied in only two monkeys, since the technical difficulties were great. The determinations were made at night on fasting animals which had been given small doses of nembutal (6 mg. per kg. of body weight). This procedure is indispensable in the rhesus monkey and does not affect the rate of metabolism (Benedict, 1938).

Serum cholesterol was estimated by the colorimetric method of Pijoan and Walter, 1937. Frequent determinations were made from blood taken from the saphenous vein at varying periods after operation.

Creatine excretion was studied by Benedict and Myers' (1907) modification of Folin's method in 5 of the group of adult monkeys. The animals were kept in metabolism cages and 48-hour specimens of urine were collected in bottles containing a small amount of toluene as a preservative. The diet was low in creatine.

RESULTS

Adult rhesus monkeys. Consequent to thyroidectomy there was no significant change in either appearance or behavior of the animals, except in

TABLE 2. SERUM CHOLESTEROL STUDIES IN THYROIDECTOMIZED MONKEYS

MONKEY NO. AND SEX	NO. OF DETERMINATIONS	PREOPERATIVE RANGE	MEAN	NO. OF DETERMINATIONS	POSTOPERATIVE RANGE	MEAN
		mg. %	mg. %		mg. %	mg. %
671 F	5	117–148	128	6	95 to 156	116
696 F	5	90–153	123	3	108 to 147	129
697 F	7	99–150	123	9	117 to 145	128
701 F	7	95–130	111	8	82 to 138	109
S 5 M	5	117–137	127	5	113 to 140	125
S 6 M	4	126–150	137	4	126 to 145	138

monkey S 5, which looked exceedingly pale. The thyroidectomized animals were quite as active as the unoperated controls. There was no evidence of sluggishness or loss of appetite. The body weight did not change markedly. In *monkey 701*, which survived the operation for 15 months, the body weight was 4.45 kg. at the time of thyroidectomy, 4.45 kg. 3 months later and 3.98 kg. 10 months after removal of the thyroid gland. The survival time varied from 44 to 487 days. Except in *monkey S 6*, which was not autopsied, widespread tuberculosis was revealed. This was a common occurrence in our colony.

The serum cholesterol in the animals before operation ranged from 90 to 153 mg. per cent, with a mean of 123.9 ± 2.0; after operation it ranged from 95 to 156 mg. per cent, with a mean of 123.5 ± 2.0. In 100 determinations on normal adult female rhesus monkeys Hartman and Fleischmann (1941) found serum cholesterol values ranging from 85 to 191 mg. per cent, with a mean of 123 mg. per cent (table 2).

Statistical analysis of these data shows that the differences between the values of serum cholesterol before and after operation do not even approach the limits of statistical significance.

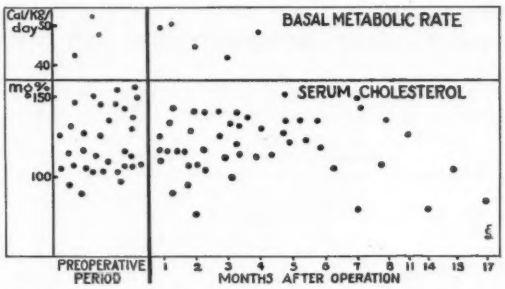

Fig. 1. Serum cholesterol and B.M.R. before and after thyroidectomy.

No changes in the B.M.R. were noted in the 2 animals on which such determinations were made. In *monkey 701*, the B.M.R. was 42.9 calories per kg. per 24 hours before operation, and 41.9 calories 3 months after operation. In *monkey 697*, two pre-operative determinations were 46.9 and 50.7 calories (mean 48.8 calories); four determinations during the first 4 months following thyroidectomy ranged from 44.1 to 48.6 calories (mean 47.3 calories per kg. per 24 hours). In figure 1, all single determinations of B.M.R. and serum cholesterol are recorded as dots.

No significant change was found in creatine excretion. The data listed on table 3 represent daily averages of periods of one week.

The data on serum cholesterol, basal metabolic rate and creatine excretion in the adult rhesus monkey show that no significant change was brought

Table 3. Daily average creatine output of thyroidectomized monkeys

Monkey number	Before operation creatine, mg.	After operation	
		Months	Creatine, mg.
671	28	2	28
697	8	1	15
697		4	15
701	7	2	7
701		5	9
S 5	15	2	12
		3	7
S 6	11	2	12

about through the removal of the thyroid gland, either shortly after operation or after long periods.

Young rhesus monkeys. Two infant monkeys were thyroidectomized at the age of about 8 months. One of these (*S 7*) survived the operation only one week. The other (*S 8*) survived for 21 weeks, when it was killed inadvertently by an overdose of nembutal. Autopsy revealed no indication of tuberculosis. Following operation the behavior of this monkey did not differ noticeably from that of normal infant monkeys. It continued to nurse well, and in addition ate some of the food given to the mother. There was no change in appearance except for an increase in weight. There was, however, a cessation of growth as shown in table 4.

During this period, judging from Schultz's tables (1933), one would expect

TABLE 4. WEIGHT AND HEIGHT OF THE INFANT RHESUS MONKEY NO. S 8

	AT OPERATION (ABOUT 8 MO. OLD)	16 WEEKS AFTER OPERATION	21 WEEKS AFTER OPERATION
Weight, gm.	1140	1450	1474
Sitting height, mm.	282	281	280
Head length, mm.	76	75	76
Foot length, mm.	101	101	101

an increase of 25 to 30 mm. in sitting height and a slight increase (about 5 mm.) in foot length. Head length shows, on an average, in normal animals no significant increase during this period. The weight increase actually was scarcely more than that which would be expected in two months in the normal of this age. Since there was complete cessation of growth, this increase in weight probably was due to metabolic changes brought about by the removal of the thyroid.

In both of the infant monkeys the serum cholesterol showed a sharp rise immediately after operation. In *S 7* it rose from 160 to 244 mg. per cent in the one week during which the animal survived. The level of cholesterol in the serum of *monkey S 8* showed a corresponding rise, reaching a peak of 326 mg. per cent 5 weeks after operation. Marked fluctuations quite similar to those observed in hypothyroid children and thyroidectomized adult rabbits followed this rise (fig. 2).

In addition to these 2 infant rhesus monkeys, a young male of about 2 years (*S 13*) was thyroidectomized. Clinically, this animal exhibited no signs of thyroid deficiency during its 4 months of survival. Serum cholesterol showed a transient rise to 218 mg. per cent, which is slightly above the range of normal.

Demonstration of Completeness of Thyroidectomy

It is of importance to show that the failure of thyroidectomy to produce any effect on the adult rhesus monkeys was not due to incomplete removal of the thyroid gland or to the presence of accessory thyroid tissue. Autopsies, therefore, were performed on all animals except one (*S 6*) which was lost

in another experiment. In addition to gross examination, all material even slightly suggesting thyroid tissue, was examined microscopically. In one adult animal (*S 5*), a careful study of mediastinum, neck (including larynx) and the pharynx including the tongue, was made; all tissue was sectioned serially and stained with hematoxylin and eosin. No trace of thyroid tissue was found postoperatively in any animal.

During life, the monkeys were injected with thyrotropic hormone of the pituitary to determine whether any thyroid tissue capable of responding to this factor was present. This method had been useful in gathering information on the presence or absence of functional thyroid tissue in dwarfed children

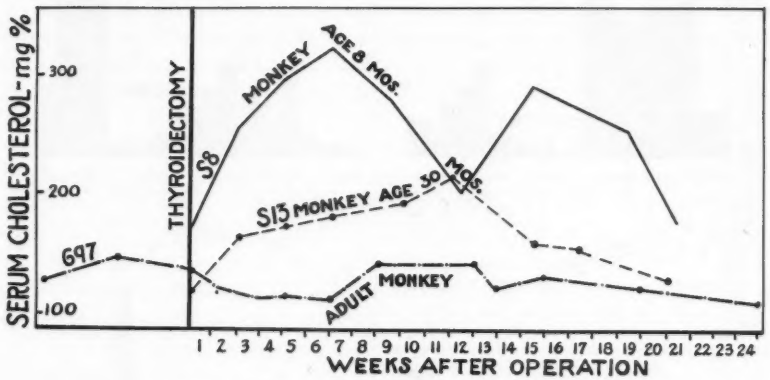

Fig. 2. Serum cholesterol levels following thyroidectomy.

(Wilkins and Fleischmann 1941). As the technical difficulties of making repeated determinations of the basal metabolic rate in the rhesus monkey were too great, we confined ourselves to a study of the effect on serum cholesterol and urinary creatine, as had been done in our work with children. In the latter, we had found an increase in creatine excretion to be the most sensitive measurement of thyroid effect.

The urinary creatine was determined daily, the serum cholesterol at irregular intervals. The base level of creatine excretion was measured during an untreated control period for a number of days. This was followed by a 10-day period of treatment with thyrotropic hormone during which 1 cc. (50 units) of the anterior hypophyseal thyrotropic factor[2] was injected daily. Following the administration of thyrotropin, no treatment was given until the creatine excretion had fallen to the base level. Each animal then was tested with a single dose of 1 mg. of thyroxin. The injections of thyrotropin were begun 5 months (*monkey 701*), two months (*monkey S 5*) and one month (*monkey S 6*) after operation, respectively. In figure 3, two of these experiments and two control experiments in the same animals before operation are

[2] We wish to thank Dr. A. Stanley Cook, of Ayerst, McKenna and Harrison, Rouses Pt., N. Y., for furnishing the thyrotropic factor.

charted. From this chart it is clear that thyrotropin produced a great increase in creatine excretion only when tested before operation, whereas it evoked no response in thyroidectomized animals.[3] Administration of thyroxin, 1 mg., however, was followed by an increased creatine output both before and after operation. This indicates that following thyroidectomy there was either

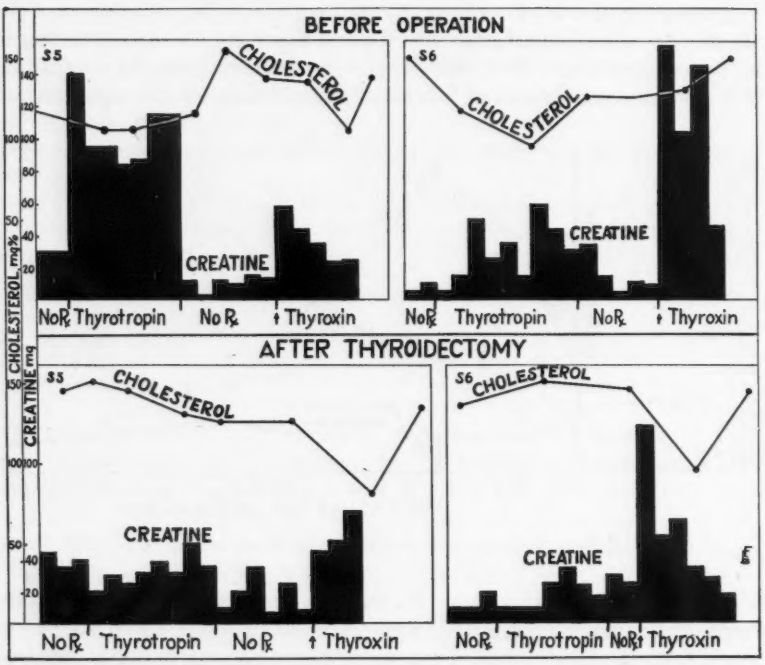

Fig. 3. Response of serum cholesterol and creatine excretion to thyrotropin and to thyroxin.

no thyroid tissue left or only an amount insufficient to cause measurable effect after stimulation with thyrotropin.

DISCUSSION

The data presented may help to clarify the conflicting reports of the older literature. Although our material is small, it shows that the age factor is of importance in determining the effect of thyroidectomy in the rhesus monkey. A survey of the older literature reveals the possibility that those authors who reported positive results used either younger animals or species

[3] The interval between the two courses of treatment with thyrotropin in *monkey S 5* and *S 6* was 3 months. This time interval was considered sufficient to eliminate the possible inhibiting influence of antihormones on the response to the second course of treatment. Presence of antihormones can be definitely ruled out, however, as explanation for the lack of metabolic response to thyrotropin in the thyroidectomized rhesus *monkey 701*, as this animal had received no previous treatment with thyrotropin.

other than those who observed that thyroidectomy had no effect on the 'monkey.'

The observations on the rhesus monkey are in close agreement with those of Simpson (1913) on sheep. This author emphasized that age has an important influence on the effects of thyroidectomy in this animal. In adult sheep he found no signs resembling myxedema of man except for slight slowing of the pulse in some animals. No lowering of the body temperature and no tendency for the wool to fall off were noted. The sheep remained active and gave birth to full-term, healthy lambs which they suckled. When thyroidectomy was performed on lambs, however, typical cretins resulted. There was a marked fall in body temperature and the wool became coarse and rough.

It is of interest that the influence of age on the effects of thyroidectomy is of importance in some species, as for example, the sheep and the rhesus monkey, whereas in other species such as man, the effects of thyroidectomy on metabolism and activity do not depend upon the age at the time of operation.

As we had stressed especially the absence of hypercholesterolemia following thyroidectomy in our adult monkeys, it should be remembered that in man incomplete thyroidectomy is nearly invariably followed by hypercholesterolemia (Hurxthal and Simpson, 1941). The rise of serum cholesterol occurs not only when toxic goiters are removed but when the normal thyroid gland is removed in the treatment of chronic heart disease (Hurxthal and Simpson, 1941; Gilligan, Volk, Davis and Blumgart, 1934). Our experiments, therefore, indicate that there is a difference between the effects of the removal of the normal thyroid glands in adult man and in the adult rhesus monkey.

It is of interest that the rhesus monkey is also comparatively insensitive to removal of other endocrine organs. Collip, Selye and Neufeld (1937), and Chapman and Fulton (1938) have shown that this species of monkey does not die in diabetic coma after total pancreatectomy. The metabolic disturbance following removal of the pancreas is much milder in the rhesus monkey than in either dog or cat. Mirsky and his associates (1941) have, however, reported the occurrence of the symptoms of diabetic acidosis after withdrawal of insulin and food in the depancreatinized rhesus monkey.

Since the thyroid gland of the adult rhesus monkey shows histologic signs of activity, it is difficult to assume that it has lost its part in the regulation of metabolism. It seems more probable that some complementary regulatory mechanism normally is present in the adult of this species and is capable of taking over the function of the gland after its removal.

SUMMARY

In 6 adult rhesus monkeys total thyroidectomy had no effect on their appearance, activity or serum cholesterol level. In two of these animals, the B.M.R. was estimated before and after removal of the thyroid and showed no change. The animals survived the thyroidectomy from 44 to 487 days. The completeness of the thyroidectomy was demonstrated both by anatomic search and by physiologic tests with thyrotropin. In addition, two infant

rhesus monkeys were thyroidectomized at the age of about 8 months. Both animals showed a sharp rise of serum cholesterol following thyroidectomy The level of serum cholesterol in the longer surviving infant monkey reached a peak of 326 mg. per cent 5 weeks after operation. Complete cessation of growth also was observed in this animal. These data suggest that the age factor is of great importance in determining the effect of thryoidectomy in the rhesus monkey.

We are indebted to Dr. Carl G. Hartman and to Dr. Lawson Wilkins for their valuable advice and criticism; to Dr. Christopher Tietze for his help with the statistical evaluation of our data, and to Mr. Karl Koudelka for carrying out the determinations of the B.M.R.

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EFFECTS OF INSULIN ON THE BLOOD LIPIDS OF MAN

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IT IS WELL KNOWN that increased amounts of lipid appear in the blood of the uncontrolled diabetic subject (Bloor, 1921; Allen, 1922; Gray, 1924; Blix, 1925; Bloor *et al.*, 1927). The recognition, however, that the pancreas contains a factor, other than insulin, that influences lipid metabolism led to a recent re-investigation of the rôle of insulin deficiency upon the level of the blood lipids. It was shown by Gibbs and Chaikoff (1941), that hypoinsulinism induced in the dog by excision of the pancreas can produce under certain experimental conditions an increase in the triglyceride content of the blood. Although the levels of cholesterol and phospholipid were also increased under these conditions, the increases were not as striking as those in fatty acids, and in many cases rises in fatty acids were unaccompanied by increases in phospholipid or cholesterol.

The effect of hyperinsulinism upon lipid metabolism is not clear. Page *et al.* (1931) have reported that serum phospholipids of rabbits decreased by about 30 per cent after the injection of large doses of insulin that induced convulsions; the other lipid constituents were affected to a smaller degree. Randall *et al.* (1938) found no change in the levels of various lipid constituents in the blood of human subjects who had been subjected to repeated insulin shocks. No significant changes in phospholipids, total cholesterol, total fatty acids and total lipids of the liver, kidney, brain or heart have been observed in rabbits that received a single convulsive dose of insulin (Page *et al.*, 1931) or in those that had been subjected to repeated insulin convulsions (Randall, 1940).

The introduction of insulin-shock in the treatment of schizophrenia provided an opportunity for investigating the effects of severe and prolonged hyperinsulinism upon the blood lipids of man. In the present study the levels of blood cholesterol, both free and esterified, total fatty acids, and phospholipids were measured in 9 subjects in whom hypoglycemia was maintained for several hours by the administration of as much as 460 units of insulin.

EXPERIMENTAL PROCEDURE

The subjects used in this study were under treatment for schizophrenia. The initial injection of insulin in each patient was 20 U; the amount administered thereafter was increased by 20 U daily until shock resulted. The amount of insulin injected on subsequent days was then varied by 10 to 20

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TABLE 1. EFFECT OF INSULIN SHOCK ON WHOLE BLOOD LIPIDS OF MAN

CASE	TIME AFTER INSULIN	STAGE OF SHOCK*	BLOOD SUGAR	WHOLE BLOOD LIPIDS (MG. PER 100 CC.)					
				CHOLESTEROL			TOTAL FATTY ACIDS	PHOSPHOLIPIDS	TOTAL LIPIDS
				TOTAL	FREE	ESTER			
	minutes								
Patient S1, Male	0	a	102	197	99	98	435	290	632
Insulin injected, 200 U	92	b	28	217	95	122	416	310	633
	191	c	18	214	101	113	440	293	654
	212	d	17	197	101	96	487	292	684
	239	d	20	194	97	97	470	302	664
	259 (14)[1]	f	89	204	97	107	432	308	636
Patient S2, Female	0	a	99	220	110	110	409	300	629
Insulin injected, 110 U	114	c	14	225	110	115	392	302	617
	174	d	15	205	101	104	433	355	638
	101	d	25	232	115	117	501	355	733
	255 (23)[2]	f	84	215	106	109	395	296	610
Patient S3,[3] Female	0	a	107	188	106	82	446	300	634
Previous insulin, 57 days	77	c	28	208	92	116	445	284	653
Previous insulin shocks, 41	139	d	47	188	105	83	458	310	646
Insulin injected, 230 U	181	d	22	206	117	89	450	334	656
	236 (45)[2]	f	118	210	115	95	441	311	651
Patient S4, Female	17	—	111	170	105	65	419	278	589
Previous insulin, 97 days	171	c	27	172	105	67	430	251	602
Previous insulin shocks, 34	215	d	39	170	110	60	423	283	593
Insulin injected, 460 U	237	d	—	170	100	70	402	251	572
	273 (33)[2]	f	105	178	105	63	447	289	625
Patient S5, Female	0	a	145	187	97	90	353	243	540
Previous insulin, 18 days	104	c	15	189	100	89	352	221	541
Previous insulin shocks, 11	134	d	17	214	120	94	372	243	586
Insulin injected, 120 U	204	e	17	188	100	88	383	230	571
	249 (36)[2]	f	108	187	100	87	416	290	603
Patient S6,[4] Male	0	a	103	164	113	51	335	232	499
Previous insulin, 48 days	134	c	70	160	110	50	347	286	507
Previous insulin shocks, 35	181	c	20	175	106	69	313	283	488
Insulin injected, 320 U	221	c	22	184	106	78	305	316	489
	240 (11)[1]	f	109	178	100	78	313	265	491
Patient S7, Female	0	a	99	155	83	72	415	307	570
Previous insulin, 27 days	116	c	21	160	99	61	400	357	560
Previous insulin shocks, 13	239	c	27	160	99	61	431	320	591
Insulin injected, 300 U	278 (34)[1]	f	160	145	91	64	437	318	582
Patient S8, Male	0	a	85	120	70	50	359	351	479
Previous insulin, 55 days	156	c	25	120	70	50	332	307	452
Previous insulin shocks, 43	214	c	23	126	70	56	379	335	505
Insulin injected, 190 U	238 (21)[1]	f	71	137	75	62	363	355	500
Patient S9, Male	0	a	92	148	87	61	413	337	561
Previous insulin, 11 days	108	c	28	163	88	75	346	300	509
Previous insulin shocks, 2	140	c	60	163	95	68	350	272	513
Insulin injected, 150 U	216	d	28	160	85	75	352	280	512
	247 (25)[1]	f	90	166	89	77	352	304	518

* The following is employed to describe the stage of shock: *a*, the stage immediately before the injection of insulin; *b*, clouded consciousness; *c*, stupor or loss of contact; *d*, suppression of pyramidal function; *e*, tonic extensor spasms; *f*, after termination of the shock.

[1] Figure in parentheses refers to time after injection of 100 to 160 cc. of 25 per cent glucose intravenously and the drinking of a solution containing 375 gm. of sucrose.

[2] Figure in parentheses refers to time after receiving 375 gm. of sucrose by stomach tube.

[3] Received 1 and 7/8 grains of sodium amytal 88 min. after insulin injection.

[4] Received 1 and 7/8 grains of sodium amytal 149 min. and again at 177 min. after the insulin injection.

U in order to produce the desired depth of shock. Insulin therapy was given for 5 consecutive days of the week and was omitted on Saturdays and Sundays. The patients received insulin for 11 to 97 days and had been subject to 2 to 43 shocks before lipid studies were made. They were maintained on a high caloric diet (5,000 calories). Breakfast was omitted on the days when the lipid study was made. The data are recorded in table 1.

Initial blood samples were taken from arm veins at 7:00 A.M. just before the administration of insulin. At this time the patients were in the post-absorptive state, having ingested several jelly sandwiches 12 hours previously. After the initial removal of blood, insulin was injected intramuscularly. The patients remained in bed for the entire period of observation.

The effects of insulin were manifested by a series of symptoms and signs, the various stages of which have been described by Frostig (1940). Blood samples were usually taken when a new stage appeared. The following stages describe the course of events in the shock therapy:

1. *Stupor or loss of contact.* The patient cannot be aroused by touch or call, although shaking may provoke a reaction. Stronger stimuli usually elicit flight reflexes. As the shock progresses, the patient becomes hyperactive and restless, and exhibits many primitive movements such as kissing and sucking movements and licking of lips. The forced grasp reflex is quite common.

2. Appearance of reflexes indicative of suppression of pyramidal function. These are the Babinski and Oppenheim signs. A general increase in muscle tonus occurs.

3. Tonic extensor spasms. These appear only in deep shock and occurred in only one of the patients in this series.

Shock was terminated from 3.5 to 4.5 hours after the injection of insulin. This was accomplished by means of *a*) the introduction by stomach tube of 600 cc. of a solution of fruit juice containing 375 gm. of sucrose or *b*) the intravenous injection of 100 cc. or more of 25 per cent glucose followed by the drinking of a sucrose solution. Blood samples were taken during this stage after the patient became fully conscious.

Convulsions were not permitted to occur in the patients recorded here. When convulsions were imminent (as indicated by the presence of rapid symmetrical twitchings of the face, neck or arm muscles) 1 and 7/8 grains of sodium amytal were administered intravenously. This served to prevent the onset of convulsions.

Five cc. of oxalated blood was pipetted with stirring into an Erlenmeyer flask containing 40 cc. of alcohol. The technic for extraction and determination of lipids has been described elsewhere (Chaikoff and Kaplan, 1934). Free cholesterol was determined in an acetone solution after the precipitation of the phospholipids. The proteins in 1 cc. of blood were removed by the zinc hydroxide method of Somogyi (1930). After centrifuging, the glucose in the supernatant fluid was determined by the ceric-sulfate method of Hassid (1936).

RESULTS

During the period of insulin shock, the blood sugar fell as low as 15 or 17 mg. and remained at low levels for nearly 2 hours (table 1). The depth of shock observed in these subjects was quite variable. In 3 of the subjects (*S6*, *S7*, and *S8*) who received 190 to 320 U of insulin, the degree of shock did not extend beyond the hyperactive phase during the 4 hours of observation. The other 6 subjects showed much deeper shock. In one case (*S5*) tonic extensor spasms appeared.

The almost complete removal of glucose from the blood stream failed to change appreciably the concentrations of cholesterol, fatty acids or phospholipids of the blood. The injection of 460 units of insulin had as little effect as 110 units.

SUMMARY

The injection of massive doses of insulin into human subjects failed to alter significantly the blood lipid level. The concentration of free and esterified cholesterol, total fatty acids and phospholipids in the blood were unaffected by the almost complete removal of glucose from the blood stream.

We are grateful for the cooperation of Dr. J. Schreiber and Dr. J. P. Frostig of the Stockton State Hospital.

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NON-SPECIFIC RESULTS OBTAINED WITH MICRO-METHOD FOR ASSAY OF PROLACTIN

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THE LOCAL response of the crop-sac or micro-method of testing for prolactin, *i.e.*, by intracutaneous injection over the pigeon's crop-sac, was introduced by Lyons and Page (1935). Because of its sensitivity it is currently used for detecting and estimating the prolactin content of blood and urine. The same method has been employed, with macerated tissue, in several experimental investigations of the prolactin content of pituitary, liver and other tissue. Almost nothing has been published on the reliability and specificity of this method, although this was questioned by Leblond and Noble (1937), and Bates and Riddle in 1940, showed that the volume of fluid utilized in these injections must be kept constant in order to obtain results of quantitative value.

In the present studies on this type of injection we have found that various non-pituitary products, both organic and inorganic, cause a proliferation of the crop epithelium which simulates that induced by prolactin. Our tests, therefore, have been extended to a large number and variety of substances in an attempt to learn something about the mechanism of this non-specific stimulation. Many tests were made to determine the effective amounts of certain substances (preservatives, tannic acid, histamine) which are more or less likely to be present in tissue and urine extracts subjected to this form of assay. More than 300 such tests were made in addition to many control observations. Results from 213 of these are tabulated for this report.

Since prolactin exerts its effects upon the crop epithelium by stimulating division of the epithelial cells, and since the response of the crop-sac to various substances raises question as to the specificity of this response to prolactin, measurements of the rate of cell division were made in all cases using the colchicine method. These measurements provide information which seems essential for a solution of the above-mentioned problems.

EXPERIMENTAL PROCEDURE

In all tests 0.5 cc. intracutaneous injections, and Carneau pigeons, aged 7 to 10 weeks after hatching, were used. The number of injections varied from 1 to 5, and the time of examining the crop-sac, following the last injection, varied from 2 to 48 hours. Only in the few 96-hour tests did we divide the daily dose into 5 'blebs,' as is done in Lyons' later method. When injections

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were made twice (48 and 24 hours before examination) an attempt was made to place the second injection within the site previously used, and all of the material was put into a single bleb.

All birds were injected with 0.6 mg. of colchicine 7 hours before the crop-sacs were inspected and preserved for sectioning. The usual gross estimates of the response were made against a good light and 5 grades of stimulation (0 to 4+) recorded. It was not always possible to be sure that the segment of crop epithelium preserved was that directly beneath the site of injection within the skin, nor entirely certain that all mitotic counts were made in stimulated parts of the excised segment. For these reasons the higher counts of mitotic figures following injections are more significant than are lower counts. The figure for each specimen was obtained by averaging the number of mitoses observed in 5 different fields observed under the high power of the microscope. The tables give the lowest and the highest values thus obtained from various similarly-treated crop-sacs; where an average of the figures seems important these are supplied in the text. In tests made 2, 4 and 7 hours after injection (excepting 7 hours after a fifth injection) the feathers were plucked 7 hours before the crop epithelium was removed for examination.

In many cases the height of epithelia, which had been rated as showing one or another grade of macroscopic response, was measured in the fixed preparations. Quite generally these were thicker than control (negative) epithelia.

RESULTS

Control data of various types are given in the first part of tables 1 and 2. Crop-sacs from 20 uninjected birds from which the feathers were not plucked (plucking is necessary in the usual tests) showed an average mitotic count of 7, with a range of 2 to 13. A similar average value (7.7) was obtained from 6 tests made 48 hours after feather plucking only, but in 4 tests at 7 hours after plucking the average count (13.3) was perhaps significantly higher. Two injections of distilled water had little or no effect on the mitotic count at 24 hours after the last injection (average for the 2, 13.5), but 7 hours after a single injection the count was probably significantly increased (average for 4 tests, 20.5) over the value (13.3) obtained for plucking alone. Results obtained with a single injection of sodium chloride or air (table 2) also tend to show higher mitotic counts 2, 4 and 7 hours than after 24 or 48 hours. The slight increase observed at these short periods with these control substances is ascribable in part at least to the fact that mere plucking of the feathers increases the mitotic counts made soon (7 hours) thereafter. It is probably significant, however, that all averages obtained following single injections of these control substances at 2, 4 and 7 hours, plus plucking at 7 hours, were higher than the average (13.3) obtained from plucking alone. For sodium chloride those averages were, 17.1, 17 and 16; for air, 20 and 14; and for water, 30.5. It thus appears that the splitting of the skin (wound effect) over the crop wall leads, as does plucking feathers, to a very temporary increase in the rate of cell division in the crop epithelium which is separated by at least one mm. from that wound.

Only very rarely did these control procedures or substances affect the macroscopic estimate of the state of the crop-sac; occasionally it was difficult to decide whether a trace of stimulation (±) was or was not present. Slightly increased mitotic counts were observed at 7 (and 4) hours after either *a*) wounds made by plucking feathers alone or *b*) with this combined with splitting the overlying dermis by injection of air, water or sodium chloride solution, but this effect was so slight and transitory that it did not affect the

TABLE 1. RESPONSE OF CROP-SAC EPITHELIUM TO INTRADERMAL INJECTION OF VARIOUS SUBSTANCES OVER VARIOUS PERIODS AND AT VARIOUS PERIODS AFTER A LAST INJECTION

SUBSTANCE TESTED	NUMBER OF TESTS	HOURS SINCE INJECTION		TOTAL DOSE, MG.	RESPONSE IN CROP-SACS		REACTION OF SKIN
		FIRST	LAST		MACROSCOPIC ESTIMATE, USUAL	NUMBER OF MITOSES, RANGE	
Untouched control	20	0	0	0.0	0, 0, ±, ±	2–13	0
Plucked feathers only	6	48	48	0.0		2–25	0
Plucked feathers only	4	7	7	0.0	0	7–25	0
Water, control	2	48	24	1.0 cc.	±, 0	13–14	0, 4+
Water, control	4	7	7	0.5 cc.	0, 0	10–39	0, 0
Water, control	1	96[2]	7	2.5 cc.	0	8	2+
Threaded skin and crop	2	48			4+	55–76	
Thread through skin only	1	48			?	27	
Skin over crop cut (and stitched once)	1	48			?	58	
Bile (rabbit)	1	48	48	70.0	4+	40	4+
Bile (wild duck)	1	48	48	50.0	+	51	4+
Bile (wild duck)	1	48	48	5.0	±	16	2+
Ethanol, 30%	2	48	48	150.0	4+	33–45	4+
Ethanol, 10%	1	48	48	50.0	+	36	+
Ethanol, 10%	2[1]	48	24	100.0	0, 2+	5–12	2+
Ethanol, 5%	2	48	24	50.0	+	20–28	0, +
Butanol, 5%	2	48	48	25.0	+, 3+	14–78	+
Butanol, 1%	2	48	24	10.0	+, 2+	47–51	0, +

[1] Tests made on hypophysectomized pigeons.
[2] Total of 5 injections.

usual macroscopic estimate made 24 to 96 hours later. Indeed our very few tests of this point suggest that, at 7 hours after the last of 5 injections with plucking of feathers 89 hours earlier, such an increase is no longer observed. It is also notable that with rare exceptions (water, sesame oil) the control substances produced no notable reaction of the skin itself at the site of injection.

A few tests (table 1), also in the nature of control observations, were made on local wounds produced *a*) in the crop epithelium and skin and *b*) in the skin alone over the crop-sac. Both types of wounds notably stimulated mitosis in the crop epithelium, and the macroscopic estimate of response was strongly positive 48 hours after the epithelium itself had been sutured. Similar results were obtained when wounds were made in the over-lying skin alone.

The above-described observations indicate that when wound healing within the crop epithelium proceeds continuously over a 48-hour period both the usual macroscopic estimate of response and the mitotic count are similar to those reported after local administration of low doses of prolactin.

TABLE 2. RESPONSE OF CROP-SAC EPITHELIUM TO INTRADERMAL INJECTION OF VARIOUS SUBSTANCES OVER VARIOUS PERIODS AND AT VARIOUS PERIODS AFTER A LAST INJECTION

NUMBER OF TESTS	HOURS SINCE INJECTION		TOTAL DOSE	RESPONSE IN CROP-SACS		REACTION OF SKIN
	FIRST	LAST		MACROSCOPIC ESTIMATE, USUAL	NUMBER OF MITOSES, RANGE	
			NaCl, mg.; control			
2	48	48	10.0	0	7–15	0
3	48	24	9.0		4–11	+, 0, 0
1	96*	7	22.5	0	4	0
4	7	7	4.5	0	9–25	0
3	4	4	4.5	0	12–22	0
3	2	2	4.5	0	13–17	0
			Air, cc.; control			
1	48	48	0.5	±	6	0
1	48	24	1.0	0	11	0
2	7	7	0.5	0	18–22	0
2	2	2	0.5	0	12–16	0
			Sesame oil, cc.			
4	48	48	0.5 cc.	0	4–23	0, +
1	96*	7	1.0 cc.	±	11	+
			Muscle extract, mg.			
1	48	48	10.0	±	9	0
3	48	24	10.0	2+, 3+	12–30	0, +
2[1]	48	24	10.0	±	3–8	0, 0
2	48	24	2.0	0, 2+	10–22	0
2	7	7	2.0	0	13–26	0
			Ammonium hydroxide, mg.			
3	48	48	0.5	0, +, 2+	14–51	0, 2+
4	48	48	0.25	±, +	10–37	0, +
			Sodium hydroxide, mg.			
1	48	48	0.2	0	20	4+
3	48	24	0.2	±, 2+	8–13	4+
			Acetic acid, mg.			
1	48	48	0.5	±	9	0
1	48	24	1.0	±	13	0
3	48	24	0.5	+, 2+, 3+	16–21	0
			Formaldehyde, mg.			
1	48	48	5.0	4+	?	4+
1	48	48	0.5	±	24	3+
2	7	7	0.5	±, +	18–36	+
			Pituitrin, cc.			
1	48	48	0.10	0	2	0
1	48	24	0.20	0	7	2+
1	48	24	0.02	0	10	0
2	2	2	0.20	0	5–9	0
			Cresol, mg.			
1	48	48	15.0	2+	34	2+
1	48	48	5.0	+	5	0
2	48	24	5.0	2+, 0	5–32	0
			Tannic acid, mg.			
2	48	48	4.0	3+	42–83	+
2	48	48	2.0	+	17–18	0
1	48	48	1.0	+	8	0
1	48	24	2.0	3+	24	0
1	48	24	1.0	±	13	0
2	96*	7	10.0*	0, 4+	10–29	4+
2	7	7	1.0	0, ±	15–18	0
1	2	2	1.0	0	9	0
			Pyrogallic acid, mg.			
1	48	48	10.0	4+	75	2+
1	48	48	4.0	3+	35	3+
3	48	48	2.0	2+, 3+	8–34	0, +
2	48	48	1.0	2+, +	7–10	0, +
2	96*	7	10.0*	0, 3+	13–13	4+
2	7	7	1.0	0, ±	16–22	+

TABLE 2—*Continued*

NUMBER OF TESTS	HOURS SINCE INJECTION		TOTAL DOSE	RESPONSE IN CROP-SACS		REACTION OF SKIN
	FIRST	LAST		MACROSCOPIC ESTIMATE, USUAL	NUMBER OF MITOSES, RANGE	
			Sodium glycocholate, mg.			
1	48	48	10.0	4+	27	4+
1	48	48	4.0	3+	32	2+
1	48	48	2.0	2+	19	+
1	48	48	1.0	±	16	+
2	96*	7	10.0	2+, +	9–11	+
			Sodium taurocholate, mg.			
1	48	48	20.0	4+	49	4+
1	48	48	10.0	4+	8	4+
1	48	48	4.0	4+	22	3+
1	48	48	2.0	4+	9	+
1	48	48	1.0	3+	15	+
2	96*	7	10.0	2+, +	8–11	+
			Prolactin, No. 437, mg.			
1	48	48	0.008	4+	73	0
1	48	48	0.002	3+	53	0
2	48	48	0.001	2+, +	12–21	0, +
3[1]	48	24	0.001	3+	22–88	0
1[1]	48	24	0.002	4+	64	0
4	48	24	0.002	3+	30–68	0, +
4	7	7	0.002	0, 0, 0, ±	18–34	0
			Histamine, mg.			
1	48	2[2]	3.0	2+	23	2+
2	48	24	2.0	±, 4+	16–26	2+
1	48	48	1.0	+	13	2+
1	48	48	0.1	0	12	0
2	4	4	1.0	0	10–13	0
3	7	7	1.0	0	7–37	0
2	7	7	0.4	0	9–9	0
2	7	7	0.1	0	4–12	0
1	96*	7	5.0	+	16	3+
2	2	2	1.0	0, +	21–29	0
			Leukotaxine, mg.			
2	48	48	2.0	±	7–12	+
2	48	48	1.0	2+, 0	4–13	2+, 0
1	48	48	0.1	+	5	+
2	30	30	1.0	±	12–17	3+
2	24	24	1.0	±	21–21	3+
3	7	7	1.0	+, ±, 0	27–34	0
2	4	4	1.0	±	14–31	0
2	7	7	0.4	0	12–24	0
2	7	7	0.1	0	11–14	0
1	96*	7	5.0	2+	7	2+
1	96*	7	2.5	+	20	2+
2	2	2	1.0	0, ±	31–32	0

* Total of 5 injections
[1] Tests made on hypophysectomized pigeons
[2] Total of 3 injections

Both the plucking of feathers and the injection of substances such as water, sodium chloride and air (in the amounts used) usually cause a transient increase in the mitotic count; but such 'wound effects' have little or no effect on macroscopic estimates made 24 or 48 hours later. In contrast, however, at 48 (or 24) hours after their injection, many substances were observed to produce an increase in the mitotic count, a positive macroscopic response, and a notable skin reaction. Among such substances are: ammonium hydrox-

ide, acetic acid, tannic acid, pyrogallic acid, ethanol, butanol, formaldehyde, cresol, bile, sodium glycocholate and sodium taurocholate (table 1 and 2).

It is obvious that even qualitative tests for prolactin may be vitiated by the presence of adequate amounts of any one of the above-mentioned substances in the material injected. For example, in our own few tests on the prolactin content of urine we obtained positive responses at dosage levels of 0.4 to 2.0 mg. of tannate precipitates from urine. But 1 to 2 mg. of tannic acid alone gives a positive result (table 2).

Fairly distinct from the effects produced by the substances listed above, although like them in giving macroscopic responses and skin reactions at 48 hours, are leukotaxine[1] and histamine. These showed little or no ability to increase mitotic rate at 48 hours after injection (table 2). These data appear to indicate that when wound substance, leukotaxine (Menken 1940), is added to the wound produced by plucking and splitting the skin (by injection), the increased mitotic rate persists somewhat longer—certainly for as much as 24 hours (unlike water, air, sodium chloride)—but not for a 48-hour period. Increased mitotic rates observed at this 48-hour period may thus be regarded as the result of a wound substance continuously produced in the skin as a result of the presence there of an irritant, or of tissue damage produced by the irritant. Again, the apparent discrepancy observed in our tables between a macroscopic response and mitotic count rests on the fact that the irritating agent had acted long enough to cause visible thickening of the epithelium, but had become inactive before the end of the test. Thus, at 48 hours after a single injection of 1 mg. or less of each of several substances (tannic, pyrogallic and acetic acids; sodium taurocholate; leukotaxine; table 2) a positive macroscopic response was observed, together with a normal mitotic count. Leukotaxine and histamine caused a very prompt stimulus (at 2 hours) to cell division, but in one test tannic acid failed to do so; the dose was 1 mg. in each of these tests. Little or no effect at 2 hours was produced by the injection of control substances, sodium chloride and air.

Pituitrin notably failed to produce either of the three effects commonly produced by the above list of substances (table 2). Ten mg. of a dried extract of beef muscle, obtained from extraction at pH 9 to 10 for several hours and precipitation by ethanol (sp. gr. 0.84), produced little reaction in the skin. It was effective, however, in producing the macroscopic response, and—except in 2 hypophysectomized birds—it apparently increased the mitotic rate.

One important group of observations is not given in the tables. Single injections of 30 per cent ethanol, 3 per cent cresol, 1 per cent formaldehyde or 1 per cent ammonium hydroxide completely eroded or destroyed the epithelium of that part of the crop-sac which was immediately beneath the site of the injection. These destructive concentrations are only 3 to 10 times more than the minimum stimulating dose. Probably larger amounts of most of the other chemical agents tested would produce local destruction of epithelium, but this is not true of prolactin. The mitotic rate of the highly proliferated tissue around the margin of the damaged area was similar to that obtained

[1] The leukotaxine was supplied by Dr. Valy Menken, Harvard Medical School.

when the crop wall was threaded. In these tests the skin around the site of injection was always highly edematous, but it was not perforated as was the crop-sac mucosa.

When, therefore, one notes that, in contrast to prolactin, the several non-specific substances exert their action through tissue injury, and that relatively large amounts of those substances are required to produce this mimicry of the prolactin effect, the specificity of the local crop-sac response to prolactin is not left in doubt. Prolactin acts locally in minute amounts when there is little evidence of skin injury, and it acts when injected at sites far removed from the crop-sacs. Cell proliferation following a wound or injury is of course the expected result in tissues generally. In the cases considered in this study, however, the primary wound is in the skin and only secondarily in the responding crop-sac tissue.

DISCUSSION

Liver tissue of sub-mammalian classes, and the brain tissue of certain fishes, were observed by Leblond and Noble (1937) to produce in 48 hours or less apparently positive macroscopic responses in this type of test; such responses, however, were thought to differ from those produced by prolactin. It was stated that the epithelia in these tests (and also in those using local implants of pituitaries of all vertebrate classes) contain few or no fat granules stainable with Scharlach R. For this reason these 'positive' responses were not interpreted as a response to prolactin. We have not fully investigated the possible significance of the reported absence of fat droplets in epithelia subjected to these short-term tests, but under the same experimental conditions we find that butanol, the only substance thus tested, does produce fat droplets stainable with Scharlach R. Our results with bile and bile salts clearly suggest that the results obtained by Leblond and Noble with liver tissue were of the non-specific type exemplified by the action of alkali and cresol.

Lyons has illustrated with photomicrographs the thickening of crop epithelia subjected to 48-hour local tests (1937, *a*) and to 96-hour local tests (1937, *b*), but only when purified prolactin was used. In this last-named paper Lyons claims that the micro-test is able to detect 0.01 microgram, or one ten-thousandth of a macro-unit. It may be noted that the method finally adopted by Lyons (1937, *b*) involves the making of 5 blebs (0.1 cc. each) over wide areas of the crop daily on 4 successive days. Perhaps two factors in this later or modified method contribute to giving it maximum sensitivity. *a*) As modified, the method should produce a relatively larger amount of 'wounding'; and, if these successive wound effects do not quite rate a lowest positive response, they may render it possible for the smallest trace of prolactin to convert this into a macroscopic response. *b*), Dilution of the dose of prolactin for injection on 4 instead of 1 day should increase the effectiveness of the dose since, in 48-hour tests, Bates and Riddle (1940) found that a minimum stimulating dose of prolactin in 0.05 cc. of solution has its apparent effectiveness increased 4 times by a 10-fold dilution.

This last-named result was noted by Bates and Riddle to indicate that

local stimulation of the crop-sac is not due to uncomplicated diffusion of prolactin. They suggested that in response to the irritation produced by the injection, some substance released in the skin serves to augment the effect of the prolactin. This view has apparently been established by the results of the present study. Especially decisive is the observation that as little as 1 mg. of leukotaxine causes an immediate (at 2, 4 and 7 hours) increase in the rate of cell division in the crop epithelium.

Leukotaxine also tends to produce macroscopic stimulation at 24 or 48 hours after the last previous injection, but the mitotic count is normal at 48 hours. Again, this demonstration that a wound substance shares in the production of this response, and that its stimulus to cell division is immediate and not prolonged, are facts which support the view that the entire list of non-specific substances exert their action upon the crop epithelium as a secondary result of their excitation of 'wound substance' in or near the lining of the crop.

The various substances here found capable of producing non-specific stimulation of the crop-sac in 48-hour tests all require 1 to 10 mg. of material for effective stimulation, but under similar conditions prolactin (*No.* 437, 7 I.U. per mg.) is effective in dosage levels of less than 1 microgram. Since this is less than one-thousandth of the required amount of non-specific material the degree of the response to prolactin is, therefore, a highly specific one. Of similar meaning is the fact that prolactin is effective, while probably all of these non-specific substances are ineffective when injected at sites far removed from the crop-sacs.

SUMMARY

Plucking feathers incident to making local crop-sac tests for prolactin leads to a slight, temporary (2–7 hours) increase in the rate of cell division in the underlying epithelium of the crop. A further slight and temporary increase in mitotic rate in this epithelium is produced by splitting the skin or by intradermal injections of 0.5 cc. of control substances such as air, water and 0.9 per cent sodium chloride solution. This temporarily increased mitotic rate is compatible with successful use of the method, but pronounced wounding of the overlying skin, and also the presence of irritants in the material injected, induce a prolonged increase (24–48 hours) of mitotic rate together with a positive macroscopic response.

Many substances tested at 48 hours after their first injection are apparent irritants since they produce a positive macroscopic response together with an increased mitotic count. Such substances, and the smallest effective dose (usually a divided dose) observed by us, are: acids (acetic, 0.5 mg.; tannic, 2.0 mg.; pyrogallic, 2.0 mg.; hydrochloric, 0.18 mg.); ammonium hydroxide, 0.25 mg.; ethanol, 50 mg.; acetone, 50 mg.; butanol, 25 mg.; cresol, 5 mg.; formaldehyde, 1.0 mg.; bile, 5–50 mg.; sodium glycocholate, 1–4 mg.; sodium taurocholate, 1–4 mg.; hydroquinone, 2–10 mg.; histamine, 2 mg.; androsterone, 1–3 mg. It is obvious that the material used for routine tests for prolactin must not contain any of these substances in the quantities indicated. Nega-

tive results were obtained with estrone, 0.5 mg., and stilbestrol, 4.5 mg. Doses of 1 to 2 mg. of the wound substance, leukotaxine, produced a prompt increase in the rate of mitosis observed at 2 to 24 hours but not at 48 hours thereafter; slight or questionably positive macroscopic responses were observed at 24 and 48 hours after injection.

The great sensitivity of the micro-test for prolactin probably depends in part upon the fact that incidental wound effects fall only slightly below a positive response.

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REACTION OF CHORIONIC GONADOTROPIN WITH PHENYLISOCYANATE

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EVIDENCE HAS been presented by Hopkins and Wormall (1933), and Gaunt and Wormall (1936), which indicated that, under certain mild conditions of reaction, phenylisocyanate reacts only with the free amino group of proteins, and the conditions of this reaction have been found to produce inactivation of the pregnant mare-serum gonadotropin (Evans, et al., 1942). This evidence has, however, been considerably weakened by recent work on the tobacco mosaic virus in which hydroxy-groups of tyrosine were readily covered by reaction with phenylisocyanate (Miller and Stanley, 1941). In view of the disagreement concerning the essentiality of free amino groups for the physiologic activity of chorionic gonadotropin (Li, et al., 1939, 1940; Bischoff, 1941, 1942) we have extended the study of the phenylisocyanate reaction to chorionic gonadotropin under the specifically mild conditions of reaction as described by Hopkins and Wormall (1933) and by Evans, Nelson, and Cartland (1942).

EXPERIMENTAL PROCEDURE

Assays for chorionic gonadotropin content were made by a method dependent upon uterine hypertrophy in not less than 9 rats per dosage level. Immature 22-day-old female rats received 3 injections, once daily, and were autopsied 76 hours after the initial dose. The reference assay curve is given in *experiment* 1, table 1. In assaying the product of the isocyanate reaction an aliquot of untreated chorionic gonadotropin, subjected to the same conditions of temperature and pH, was assayed simultaneously using littermate animals. The hormone assayed 150 international units per mg. and was prepared in this laboratory from urine of pregnancy by fractional alcohol precipitation. The amount of phenylisocyanate used corresponded to 2 to 3 mg. per cc. of reaction mixture, that of chorionic gonadotropin to 0.2 to 0.4 mg. per cc. Phosphate buffers were used to control the pH of reaction. The phenylisocyanate was added in two increments and the time of reaction, as judged by the disappearance of the characteristic powerful phenylisocyanate odor, was approximately 1 hour. The maximum temperature of reaction is recorded in table 1. The reaction mixture was subjected to constant stirring during the period of reaction and the temperature was controlled by immersion of the vessel in a salt-ice bath. Further details of reaction and assay are given in

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table 1. In *experiments* 2 and 3, the filtrates of the reaction product were administered to the assay animals. In *experiment* 4, the whole reaction product, including diphenylurea, was administered.

RESULTS AND DISCUSSION

The results clearly establish that chorionic gonadotropin, like the mare-serum hormone, is more than 90 per cent inactivated by phenylisocyanate under conditions which, up to the time of the work of Miller and Stanley in 1941, have been regarded as specific for amino group reaction.

In summing up the evidence to date regarding the relationship of the amino group to the activity of the gonadotropins tested, it will be observed that *a*) acetylation and benzoylation in mildly alkaline solution produces in-

TABLE 1. INACTIVATION OF CHORIONIC GONADOTROPIN BY PHENYLISOCYANATE AT LOW TEMPERATURES

EXP. NO.	TREATMENT OF CHORIONIC GONADOTROPIN	DOSE CHORIONIC GONADOTROPIN PER RAT	UTERINE WEIGHT MEAN ± S.E.	PER CENT RECOVERY
		mg.	*mg.*	
1	Untreated	0	16±1.0	
		0.01	26±3.5	
		0.02	63±8.4	
		0.03	99±5.7	
		0.04	113±6.2	
2	Phenylisocyanate pH 8.0, below 2°C.	0.03	18±1.3	<10
		0.06	18±0.7	
		0.14	17±1.1	
	Untreated	0.03	104±4.9	
3	Phenylisocyanate pH 7.4, below −2°C.	0.15	17±1.0	<10
	Untreated	0.03	98±6.3	
4	Phenylisocyanate pH 7.4, below 0°C.	0.10	18±0.7	<10
		0.05	18±0.9	
	Untreated	0.02	71±8.0	

activation (Maxwell and Bischoff, 1935–36; Bischoff and Long, 1936; Bischoff, 1938, 1940); *b*) the slow progressive inactivation by formaldehyde is similar (Maxwell and Bischoff, 1935–36; Bischoff, 1941); and *c*) the degree of inactivation by phenylisocyanate is similar. Chorionic gonadotropin is more resistant to the action of nitrous acid (Bischoff, 1942) and apparently is more slowly inactivated by ketene (Li, et al., 1939, 1940). These reactions can no longer be accepted as specific for the amino group of proteins. Ketene not only reacts with tyrosine but also with a third component (Miller and Stanley, 1941; Wood and Ross, 1942). While failing to establish the essentiality or nonessentiality of the amino group for physiologic activity of the gonadotropins, the results of the nitrite, methylsulfate and ketene reactions have suggested a difference in the chemical reactivity of chorionic gonadotropin as compared with the other gonadotropins. Since the results of the isocyanate reaction again fail to confirm deductions based on the ketene reaction, it

seems advisable to be conservative in drawing conclusions concerning the chemical structure of the protein hormones.

SUMMARY

More than 90 per cent of the physiologic activity of chorionic gonadotropin is destroyed by phenylisocyanate under the specifically mild conditions of reaction which inactivate the mare-serum gonadotropin and which were formerly regarded as limited to reaction with the amino group.

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THYROID-INHIBITING ACTION OF THE HYPOPHYSES OF RATS FED WITH THYROID

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IN THE RAT the hypophysis has a strong thyroid-stimulating action while its own thyroid barely responds to thyrotropic stimulation. On the other hand, the thyroid of guinea pigs weighing 150 to 200 gm. is very sensitive to the thyrotropic hormone. For this reason we have injected hypophyses from rats into guinea pigs.

In studying the thyroid-stimulating action of the hypophysis it was found that if the hypophyses of rats which were fed with desiccated thyroid were injected into guinea pigs, the thyroid gland of these animals weighed less than that of the controls and showed a flattening of the epithelium (Reforzo-Membrives, 1938; 1940). The thyroid activity of guinea pigs injected with hypophyses of thyroid-fed rats has been further studied from 3 different points of view: *a*) morphologic, by investigating the weight and histology of the thyroid gland; *b*) metabolic, by measuring the basal metabolism of the guinea pigs; and *c*) by estimating the oxidizing power of the thyroid tissue. The results are reported in this paper.

METHODS

Adult male rats were given desiccated thyroid, by mixing it in the diet or by stomach tube, in daily quantities varying from 50 to 100 mg. during periods of 22 to 32 days. A daily dose of 50 mg. for 3 weeks followed by another of 100 mg. during the 4th week may be considered as the most adequate dosage.

Rat hypophyses were suspended in saline and injected intraperitoneally into normal male guinea pigs weighing from 150 to 200 gm. Generally 2 glands were injected daily during 2 successive days. Observations were made the day following the last injection.

The histologic study of the guinea-pig thyroids was made after fixation in 10 per cent formalin and embedding in paraffin. The block was sectioned and stained with hematoxylin and eosin. The height of 10 cells was measured at the widest point of each section, the cells measured lying at equal distances from the edge. The 10 readings were averaged and recorded.

The diameter of the thyroid follicles was estimated from the average of 20 measurements made on each section, and including both the peripheral

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and central parts of the gland. When the number of observations was sufficient the standard error of the mean was calculated.

Basal metabolism was measured by the method of Haldane. Carbon dioxide and moisture elimination were determined gravimetrically, and oxygen intake by difference between the loss of weight of the animal and the preceding determinations.

From these data it is easy to compute the respiratory quotient and the caloric value of both O_2 and CO_2. Each determination lasted 3 hr. and was

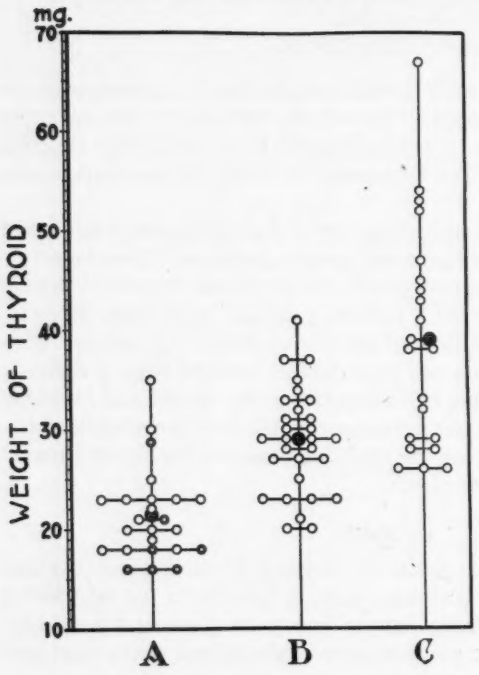

FIG. 1. Weight of guinea-pig thyroids in mg. Black circles represent average of each group. *A.* Injected with hypophyses of normal rats fed desiccated thyroid (white circles), and of thyroidectomized rats fed desiccated thyroid (double circles). *B.* Uninjected controls. *C.* Injected with hypophyses of normal rats.

performed after a 12-hour fast at a temperature of 28 to 29°C. in the interior of the chamber. Results are expressed in calories per kg. per hr.

The oxidizing power of the thyroid tissue was measured by a colorimetric method which estimates its content in oxidases. A known quantity of thyroid tissue was placed in 10 cc. of a 0.1 per cent *p*-phenylenediamine solution; after trituration it was shaken for 45 minutes at 25°C. The intensity of the color formed, read in an Evelyn colorimeter, divided by the mg. of thyroid tissue utilized, gives a value which may be considered as an index of the activity of the gland and which may be called the 'oxidation index.' Values above normal indicate an increase in activity while those below normal represent a decrease.[1]

[1] Dr. C. Galli-Mainini, whose help we deeply appreciate, performed these determinations. The detailed technique of his method will be published shortly.

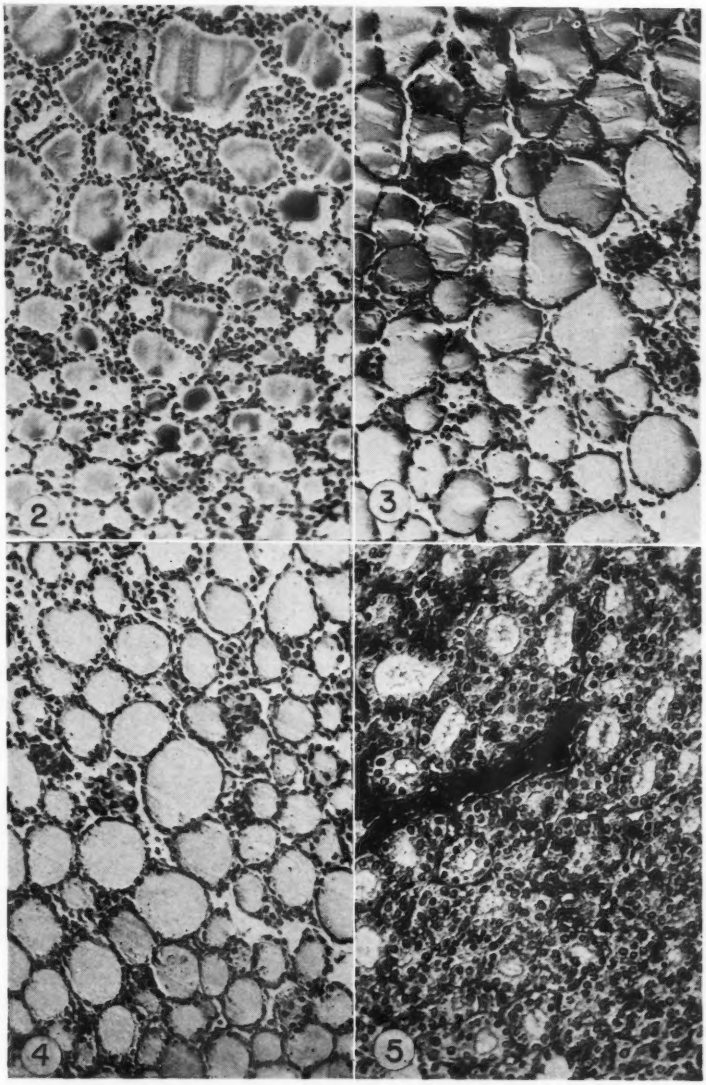

Fig. 2. Thyroid, normal guinea pig. ×190.

Fig. 3. Thyroid of guinea pig injected with hypophyses of rats fed desiccated thyroid. ×190.

Fig. 4. Thyroid of guinea pig injected with hypophyses of thyroidectomized rats fed desiccated thyroid. ×190.

Fig. 5. Thyroid of guinea pig injected with normal rat hypophyses. ×190.

RESULTS

Morphologic studies. The weight and histology of the thyroid gland were studied in 4 groups of guinea pigs. *a*) Twenty-nine normal guinea pigs of an average body weight of 179.5 ± 4.6 gm. *b*) Seventeen guinea pigs injected with hypophyses from thyroid-fed normal rats and 7 injected with hypophyses from thyroid-fed thyroidectomized rats. The average body weight of this group was 170.8 ± 2.8 gm. *c*) Twenty-one guinea pigs injected with hypophyses from normal rats fed the usual diet. Average body weight 170.4 ± 3.3 gm. *d*) Ten guinea pigs injected with various organs (liver, adrenals, spleen, testis) of thyroid-fed rats. Average body weight 176.9 ± 4.4 gm.

Weight of thyroids. In *group A* the average weight of the thyroids was 28.8 ± 0.98 mg.; weights greater than 26 mg. were found in 72 per cent of the cases. In *group B* the average was 21.4 ± 0.53 mg.; the difference (7.4 ± 1.1 mg.) is statistically significant, and represents a reduction of 26 per cent with relation to the normal controls of *group A*. In 88 per cent of the cases the weight was less than 26 mg. (fig. 1). In *group C* the average weight of the thyroids was 39 ± 2.4 mg.; the difference from values in *group A* (10.2 ± 2.57 mg.), is statistically significant, representing an increase of 35 per cent over the average of the normal controls (fig. 1). In *group D* the average was 29.6 ± 2.18 mg.; the difference in values from those in *group A* is 0.8 ± 2.39 mg., which represents a 2 per cent increase and is not statistically significant.

Histology of thyroids. The histologic appearance of thyroids of the untreated guinea pigs of *group A* was normal for animals of their weight (fig. 2). In 8 of these (average body weight 180.6 ± 4.2 gm.) the average height of the thyroid epithelium was 6.2 ± 0.27μ and the average diameter of the follicles 27.7 ± 1.67μ.

The histology of the thyroids of the guinea pigs of *group B* generally revealed characteristics of typical hypofunction (fig. 3 and 4). In 10 of these (average body weight 182.5 ± 6.5 gm.) the height of the epithelium averaged 4.6 ± 0.18μ; the difference (1.6 ± 0.33μ), is statistically significant and represents a reduction of 26 per cent as compared with *group A*. The average diameter of the follicles was 32 ± 3.83μ; the difference (4.3 ± 4.15μ), is not statistically significant, representing a slight increase (16 per cent) over that of *group A*.

Basal metabolism. Three guinea pigs were injected with hypophyses of thyroid-fed rats and the variations in basal metabolism were studied. The weights of the guinea pigs before and after the experiments were: 1, 150 and 338 gm.; 2, 269 and 290 gm.; 3, 167 and 277 gm. Before administration of hypophyses, 2 or 3 determinations of basal metabolism were made in each animal. The results may be seen in figure 6. In the 3 animals the basal metabolism decreased from 26 to 48 per cent. In *guinea-pigs 2* and 3, this decrease was observed immediately following the second injection; in *guinea-pig* 1 the lower figure was obtained 6 days after the last injection; 21 days after, when the values had returned to normal, this animal was again injected and the basal metabolism again fell to minus 38 per cent. Fifteen days later it was still minus 24 per cent.

The injection of hypophyses from normal rats does not cause a decrease in the basal metabolism of the guinea pigs. If anything, it produces an increase. *Guinea-pig* 3, which was injected with these normal hypophyses after

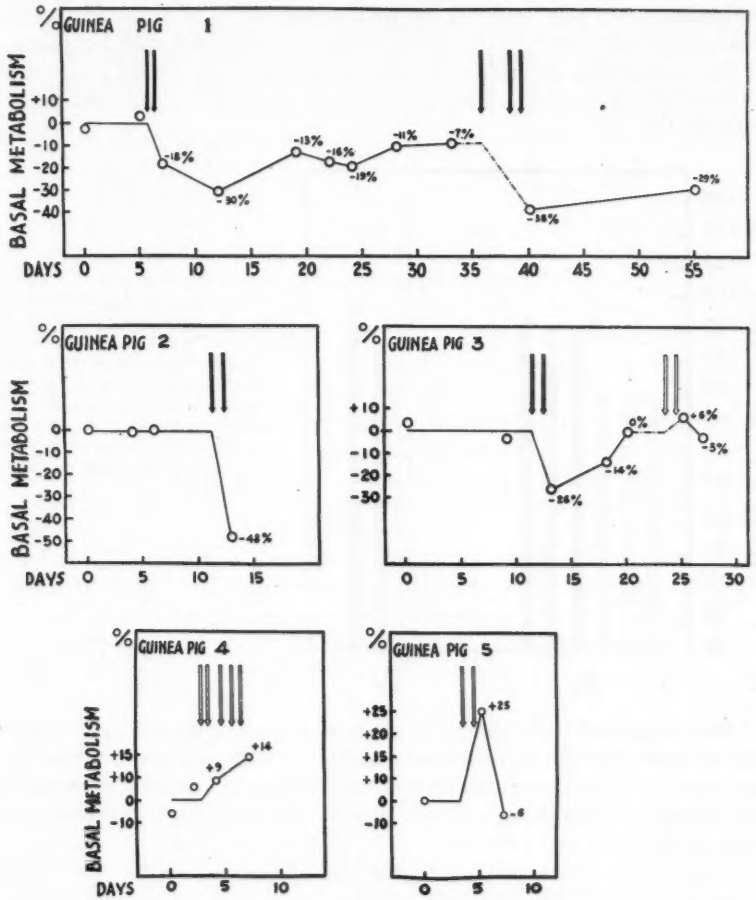

Fig. 6. Percentage change in basal metabolism of male *guinea-pigs* 1, 2, 3, 4 and 5. At each black arrow 2 hypophyses of thyroid-fed rats were injected. At each white arrow 2 hypophyses of normal rats were injected. Each determination represented by circles is expressed as percentage variation.) The line before the first injection represents the mean of the basal metabolism determinations before beginning the treatment.

its metabolism had returned to normal, showed a slight increase (plus 6 per cent). In *guinea-pig* 4 (fig. 6) the first 2 injections caused only a slight increase of plus 8 per cent; after the fifth injection the basal metabolism increased further (plus 13 per cent). In *guinea-pig* 5 basal metabolism increased to plus

25 per cent on the day following the second injection. The weights of the guinea pigs before and after the experiment were: 4, 205 and 218 gm.; 5, 182 and 206 gm.

Oxidation index. The oxidation index of the thyroid tissue of 3 normal untreated guinea pigs weighing 190 to 210 gm. was found to be 97, 100 and 104, respectively (average 100.3). In 3 guinea pigs injected with hypophyses of thyroid-fed rats, the oxidation index values were 76, 77 and 80 (average 77.7) which represents a 23 per cent decrease as compared with the normal controls (fig. 7).

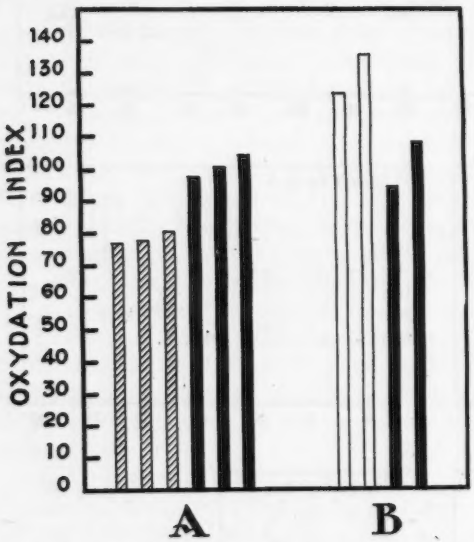

Fig. 7. Oxidation index of the thyroid expressed as arbitrary units of oxidative activity. A. Guinea pigs injected with hypophyses of thyroid-fed rats (striped columns), compared with normal controls (black columns). B. Injected with hypophyses of normal rat (white columns) compared with the normal controls (black columns).

Determinations were also made in 2 guinea pigs of similar body weight injected with hypophyses of normal rats. Values obtained were 123 and 135 (average 129) which compared with determinations simultaneously made in two uninjected controls (94 and 108; average 101) represent an increase of 28 per cent (fig. 7).

DISCUSSION

The possibility that the anterior lobe of the hypophysis may, under certain circumstances, have an inhibiting action on the thyroid has been considered by some investigators. Smith (1931) believes that there may be two thyrotropic hormones in the anterior hypophysis: one a stimulator and the other a depressor, the latter linked, perhaps, with the growth hormone. He based this opinion on experiments which demonstrated a depression of basal metabolism of rats injected daily with anterior lobe extracts for 4 months (1929) and on the fact that implantation of hypophyses repairs thyroid atrophy of hypophysectomized rats, while injections of saline extracts of the same gland have no action at all.

Studying the compensatory hypertrophy of the thyroid, Loeb and co-workers observed that oral administration of a commercial preparation of anterior lobe of the hypophysis inhibited this hypertrophy (Loeb, 1920; 1929; 1932; Loeb and Kaplan, 1924; Loeb and Basset, 1929; Gray, 1929; Loeb *et al.*, 1930). When it was found that the commercial preparation used contained large amounts of iodine (total and protein) and that inorganic iodine in those dosages had a thyroid-stimulating action, these investigators admitted that the inhibiting action observed was due to the presence of thyroid in the commercial preparation of anterior lobe (Loeb and Siebert, 1930; Closs, 1931; Loeb, 1932).

Our experiments show that, as a consequence of the oral administration of desiccated thyroid, the hypophysis develops a thyroid-inhibiting action, both in the normal and thyroidectomized rats, demonstrating that the animal's own thyroid is not necessary for this action to appear. The injection of these hypophyses into normal guinea pigs produced a decrease of 26 per cent in the weight of the thyroid and a 26 per cent reduction of the height of the thyroid epithelium, a 26 to 48 per cent decrease in basal metabolism and a 23 per cent decrease of the oxidation index of the thyroid tissue. The injection of other tissues (liver, adrenal, spleen, testis) of rats fed with thyroid had no such effect. Based on the morphologic changes observed and on the changes of the oxidation index of the thyroid, we believe that the metabolic-depressing action of the hypophysis of thyroid-fed rats, is not a direct one but is mediated through the thyroid gland.

The fact that rats fed desiccated thyroid show obvious signs of intense functional depression of their own thyroid glands (Abel *et al.*, 1925; Courrier, 1928; Gray, 1929; Cordonnier, 1929; Reforzo-Membrives, 1938; Uotila, 1940; Belasco and Murlin, 1941; Galli-Mainini, 1941) and that the hypophyses of such rats have a thyroid-inhibiting action when injected into guinea pigs, leads us to believe that the latter action may play a rôle in the physiologic regulatory mechanism between hypophysis and thyroid. An excess of thyroid hormone in the circulation, as a result, for instance, of the oral administration of dried thyroid, would act upon that mechanism in 3 ways, *a*) directly causing an inhibition of the thyroid (Uotila, 1940; Galli-Mainini, 1941); *b*) inhibiting the thyroid-stimulating action of the anterior lobe of the hypophysis (Hohlweg and Junkmann, 1933; Reforzo-Membrives, 1938); or *c*) originating or developing an active thyroid-inhibiting action in the hypophysis.

SUMMARY AND CONCLUSION

Hypophyses from *a*) normal rats fed the usual diet and *b*) normal or thyroidectomized rats fed the same diet plus desiccated thyroid, were suspended in saline and injected intraperitoneally into normal guinea pigs.

A study was made of the effect of these injections on *a*) the weight and histology of the guinea-pig thyroid; *b*) the basal metabolism of the guinea pigs as determined by the method of Haldane and *c*) the oxidyzing power of the thyroid tissue as measured by a colorimetric method which estimates its content in oxidases.

The hypophyses of normal rats fed the usual diet produced in guinea pigs the following deviations from the normal: *a*) a 35 per cent increase in the weight of the thyroid, which presented the typical histologic picture of hyperfunction; *b*) a 9 to 25 per cent increase in basal metabolism and *c*) a 28 per cent increase in the oxidation index of the thyroid tissue.

The hypophyses of normal or thyroidectomized rats fed desiccated thyroid produced *a*) a 26 per cent decrease in weight of the thyroid and a 26 per cent reduction of the height of the thyroid epithelium; *b*) a 26 to 48 per cent decrease in basal metabolism and *c*) a 23 per cent decrease of the oxidation index of the thyroid tissue.

The injection of other tissues (liver, adrenals, spleen, testis) of rats fed thyroid had no effect on the thyroid of guinea pigs.

The results obtained indicate that the hypophyses of rats fed thyroid develop a thyroid-inhibiting action which causes a decrease of the basal metabolism.

I wish to express my gratitude to Prof. B. A. Houssay for his advice and help in planning these experiments and in the supervision of the results.

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PROGONADOTROPIC AND ASPECIFIC EFFECTS OF THE SERUM OF A HORSE IMMUNIZED WITH EXTRACTS OF SHEEP PITUITARY GLANDS[1]

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IN ORDER to obtain adequate amounts of antigonadotropic serum for use in physiologic studies, it was decided to use the horse as a source of this material. Soon after the beginning of the experiments to be reported here, Thompson (1937) reported that repeated injections of sheep pituitary extracts for long periods of time resulted in the production in the horse, first, of a gonadotropic-augmenting substance, then a gonadotropic-inhibiting substance. Collip (1937), Rowlands (1938, *a*, *b*), and Katzman, Wade, and Doisy (1937, 1939) have shown that injections of homologous pituitary material produce augmenting substances in recipient animals and that inhibitory substances do not appear even after long periods of time. These augmenting substances enhance the gonadotropic activity of concomitantly administered gonadotropins. The above results are of particular immunologic interest since Collip (1937) and Selye, Collip, and Thomson (1934*b*) had reported that injections of animals with homologous gonadotropins would produce inhibitory substances and they consider their data as favoring the non-antibody nature of these substances.

Early studies (Selye, et al., 1934*a*, *b*; Gustus, et al., 1935; Kəbak, 1936) indicated that the antigonadotropic substances are relatively specific for the gonadotropic extract used in producing them. Later investigations (Rowlands, 1937; 1938*b*; Thompson and Cushing, 1937; Parkes and Rowlands, 1936), however, have shown that the degree of specificity varies considerably, and the reasons for this variation are as yet obscure. The aspecificity of the antigonadotropic substances, and the fact that their titer in the blood is apparently independent of either the precipitin or the complement fixation titers, has been interpreted as supporting the contention that these inhibitory substances are not antibodies (Collip, et al., 1940; Thomson, et al, 1941).

Since physiologic studies (Marvin and Meyer, 1941) indicate the possibility of using antigonadotropic sera for the treatment of certain pathologic

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conditions, it was of interest to study the specificity of the antigonadotropic substance produced in the horse. The results of this study are herein reported.

MATERIALS AND METHODS

The horse used was a mare pony approximately 4 years old at the time the injections were begun. Extracts of sheep pituitary glands were injected subcutaneously at varying intervals. The site of injection, the syringe, and the bottle of extract were all washed in 70 per cent alcohol before each injection to prevent sepsis. Injections were made at various places on the neck and shoulders to reduce inflammatory reactions to a minimum.

The gonadotropic extract was obtained by three consecutive aqueous extractions of acetone-dried sheep pituitary glands. The combined extracts were precipitated in four times their volume of acetone. The precipitate was dissolved in distilled water so that 1 cc. of the solution was equivalent to either 250 or 500 mg. of the original pituitary powder. The solution was supercentrifuged and the precipitate used for another purpose. The supernatant solution was filtered through a sterile Seitz filter, introduced into sterile 10 cc. vials, and stored in the refrigerator. Successive assays during the period of injection showed little if any loss of activity for as long as 5 months.

The daily dose of the extract was gradually increased from the equivalent of 0.25 to a maximum of 2.0 gm. of the sheep pituitary powder. The increase was made during the time that the serum showed augmentation (to be described later), with the hope that the appearance of the inhibitory substance might be hastened.

Blood was obtained by venipuncture of the jugular vein, and during the early part of the work was allowed to clot normally. The serum was separated by centrifugation, Seitz-filtered, and the sterile filtrate aseptically transferred to sterile bottles. The serum was kept frozen except for the short time necessary for the removal of some for use. This method gave a poor yield of serum and to increase the amount obtained, another method was devised. Briefly, the method consisted in drawing the blood into a definite amount of sodium citrate solution which prevented clotting. This mixture was allowed to stand in the refrigerator until two thirds of the volume was clear plasma. The plasma was then siphoned off, and to it was added a certain amount of calcium chloride solution which initiated the clotting process. The fibrin was separated and the serum treated as mentioned before.

The antigonadotropic activity of the serum was determined by using the 21-day-old female rat as the test animal. A total dose of 2 cc. of serum was administered in nine 0.5 cc. injections in 4.5 days; the rats were autopsied on the morning of the sixth day. The gonadotropic extract was administered in the same way as the serum, but on the opposite side of the body. The weights of the ovaries of the rats receiving the extract alone were compared with those of the rats receiving the serum and the extract. Such comparisons showed whether or not augmentation or inhibition of the action of the gonadotropic extract had occurred.

RESULTS

In table 1 are the data from assays of the pony serum obtained at intervals during the first 6 months of injections with an extract of sheep pituitary glands. The sera designated as *PN* were normal ones, obtained just before the injections were begun; the sera designated as *PA* were those obtained soon after the beginning of treatment, and the sera designated as *PX* were obtained from the pony after 2 years of injections with gonadotropic extracts. Inspection of these data indicates that the sera obtained within 95 days after the beginning of treatment augmented the extract of sheep pituitary glands concomitantly administered to immature female rats. The augmenting effect increased from the 34th day to a maximum on the 45th day, then disappeared by the 107th day of treatment. The sera obtained after the 107th day of injections inhibited the action of the gonadotropic extract used as an antigen.

TABLE 1. EFFECTS OF ANTIGONADOTROPIC HORSE SERA ON THE ACTION OF GONADOTROPIN IN EXTRACTS OF SHEEP PITUITARY GLANDS

SERUM	DAYS AFTER FIRST INJECTION	OVARIAN WEIGHTS IN MG.[1]		
		GONADOTROPIC EXTRACT ALONE	EXTRACT+ SERUM	DIFFERENCE
PN_1	0	54(4)	63(6)	+9
PN_2	0	74(3)	76(6)	+2
PA_3	7	44(2)	27(3)	−17
PA_4	22	27(3)	21(3)	−6
PA_5	34	20(2)	43(3)	+13
PA_7	45	42(3)	101(3)	+59
PA_9	95	46(3)	80(3)	+34
PA_{10}	107	49(1)	20(3)	−29
PA_{11}	118	49(1)	15(3)	−34
PA_{12}	134	67(3)	6(3)	−61
PA_{13}	183	67(3)	7(3)	−60

[1] The numbers in parentheses indicate the numbers of rats used in the assay.

Since it was desired to use the antigonadotropic serum for experimental studies, it was of interest to determine its effectiveness in inhibiting the gonadotropic activity of the pituitary glands of other species. The results of this study are included in table 2 and show several interesting facts. When the various sera were assayed against rat pituitary glands, results were obtained which show a progressive increase in the rat antigonadotropic titer. It is interesting that the inhibitory activity to rat gonadotropin was present in sera which augment sheep gonadotropin.

There are also in table 2 data which were obtained from assays of the sera against human pituitary glands. Again in this experiment, the results show a positive correlation between the antigonadotropic titer and the length of time the horse had been injected. The ability of the serum to inhibit the gonadotropic activity of human pituitary glands is of interest since it suggests the possibility of using this serum in the treatment of hypersecretion of the gonadotropic hormone in human beings. The serum also inhibited the gonadotropic material present in human urine of pregnancy.

TABLE 2. EFFECTS OF ANTIGONADOTROPIC HORSE SERUM ON THE ACTIVITY OF VARIOUS GONADOTROPINS

SERUM		GONADOTROPIN	OVARIAN WEIGHTS MG.[1]	
TYPE	DOSE, CC.	DOSE, MG.	GONADOTROPIN ALONE	GONADOTROPIN +SERUM
		Rat pituitary glands[2]		
PA_5	1.0	3 mg.	55(3)	47(2)
PA_6	1.0	3 mg.	55(3)	50(1)
PA_9	0.5	2 mg.	88(1)	31(3)
PA_9	1.0	3.5 mg.	86(3)	9(3)
PA_{10}	1.0	3.5 mg.	86(3)	9(3)
PA_{11}	1.0	3.5 mg.	86(3)	10(3)
PA_{12}	1.0	3.25 mg.	93(3)	8(3)
PA_{13}	1.0	3.25 mg.	93(3)	7(3)
PA_{12}	0.5	10 mg.	164(2)	33(2)
		Horse pituitary glands[2]		
PA_9	2.0	13 mg.	46(3)	28(3)
PA_{11}	2.0	13 mg.	46(3)	65(3)
PA_{12}	2.0	15 mg.	79(3)	58(3)
PA_{13}	2.0	15 mg.	79(3)	45(3)
PX_{10}	2.0	15 mg.	44(2)	61(3)
		Pregnant mare serum		
PA_{13}	0.5	0.25 cc.	83(2)	17(2)
PX_{10}	2.0	0.16 cc.	40(2)	18(3)
		Chorionic gonadotropin		
PX_{10}	2.0	7.5 mg.	23(2)	9(3)
		Chicken pituitary glands[2]		
PX_{10}	2.0	40 mg.	39(2)	53(3)
		Human pituitary glands[2]		
PA_4	0.5	5 mg.	21(2)	18(2)
PA_9	0.5	5 mg.	21(2)	23(3)
PA_9	2.0	8 mg.	72(2)	24(3)
PA_{11}	2.0	8 mg.	72(2)	20(3)
PA_{12}	2.0	8 mg.	72(2)	13(3)
PA_{13}	0.5	10 mg.	133(2)	20(2)
PA_{11-15}	1.0	2 mg.	31(2)	8(3)
PX_{4-7}	1.0	2 mg.	31(2)	10(3)
PX_{4-7}	2.0	1.2 mg.	26(2)	7(3)
		Hog pituitary glands[3]		
PA_{13}	0.5	$\Rightarrow$200 mg.	50(1)	97(2)
PA_{11-15}	0.5	$\Rightarrow$175 mg.	40(2)	61(2)
PA_{11-15}	1.0	$\Rightarrow$175 mg.	40(2)	53(2)
PA_{11-15}	1.5	$\Rightarrow$175 mg.	40(2)	59(2)
PA_{11-15}	2.0	$\Rightarrow$175 mg.	40(2)	36(2)
PX_{4-7}	1.0	$\Rightarrow$175 mg.	25(2)	24(2)
PX_{4-7}	1.5	$\Rightarrow$175 mg.	25(2)	32(2)
		Beef pituitary glands[3]		
PX_{10}	2.0	$\Rightarrow$300 mg.	50(2)	7(3)

Uninjected control rats had ovaries weighing 13 mg.

[1] The numbers in parentheses indicate the numbers of rats used in each assay.

[2] The gonadotropin was administered as a suspension of dried pituitary glands. The dose is the number of milligrams of dried gland given each rat.

[3] The hog and beef gonadotropins used were aqueous extracts of acetone-dried pituitary glands. The dose used is designated in equivalents of dried glands.

The gonadotropic activity of extracts of hog pituitary glands was not inhibited by concomitantly administered antiserum but an augmenting effect seems to have been produced. However, the data are so few that the differences obtained may be due entirely to chance.

The data in table 2 also show that both the early and late sera (*PA* and *PX*) inhibited the effect of the gonadotropin in the serum of pregnant mares, but not the gonadotropin in horse pituitary glands. Other reports (Selye, et al. 1934a; Severinghaus and Thompson, 1939; Meyer and Gustus, 1935; Fluhmann, 1935) have shown that repeated injections of an extract of heterologous pituitary glands decreased the size of the gonads of the recipient animal, but such was not the case in this experiment since palpation of the horse's ovaries, per rectum,[3] showed that definite changes in the size and shape of the ovaries occurred during the time anti-sheep gonadotropic activity was found.

The gonadotropic activity of the beef pituitary gland extract was augmented by the addition of 4 mg. of crystalline heme per rat to the gonadotropic solution injected at the time of assay (McShan and Meyer, 1941). The data show that not only did the antiserum inhibit the beef pituitary gonadotropin but that the endogenous rat pituitary gonadotropin was also inhibited.

The results of the assay using a suspension of powdered chicken pituitary glands show that the antiserum was unable to inhibit the effects of this gonadotropin.

DISCUSSION

The data in table 1 substantiate those obtained by Thompson (1937) who found that augmentation of the gonadotropic effects of the antigen was obtained first, and later an inhibition was demonstrated. He suggested that the augmenting activity was an expression of the presence of a substance inhibiting the action of the pituitary antagonist. No attempt has been made to test for an anti-antagonist in the serum of the horse reported here. However, results of one experiment showed that injections of augmenting serum alone produced no demonstrable effects upon the ovaries, uteri, and vaginae of normal 21-day-old female rats. The results of this experiment agree with the observations reported by Katzman, Wade, and Doisy (1937), but are not in agreement with those reported by Thompson (1937).

The antigonadotropic serum was tested for antithyrotropic activity in the adult rat. A preliminary determination showed that the extract of sheep pituitary glands used as an antigen for the horse would increase the oxygen consumption of rats when injected into them. A second series of tests showed that the antigonadotropic serum, in the doses given, failed to inhibit the increased oxygen consumption due to the antigen. However, other doses of serum or more delicate tests may yet show that the antigonadotropic serum has antithyrotropic activity.

The data in table 2 show that the sera had the ability to inhibit the gonadotropic effects of rat, sheep, human and beef pituitary glands. The

[3] The palpations were made by Dr. L. E. Casida of the Department of Genetics, University of Wisconsin.

gonadotropic activity of the serum of pregnant mares and the urine of pregnant women was also inhibited. The gonadotropic activity of horse, hog and chicken pituitary glands, however, was not inhibited. The fact that the serum inhibited the gonadotropin of pregnant mare's serum but not that of horse pituitary glands may serve as evidence for the belief that these two substances are from different sources; viz., a type of organ or source specificity.

The results of this study are interesting from an immunologic standpoint. If the antigonadotropic substance is an antibody formed in the horse by the injections of a foreign protein, its species specificity would be expected to conform to certain fundamental immunologic concepts. It has been amply shown by data obtained from agglutination, complement fixation and precipitin tests, that an antibody will react to a greater degree with its homologous antigen than it will with antigens derived from other species. The cross-reactions of antibodies with heterologous antigens have been used as a quantitative measure of the relationship between groups of related plants and animal.

Several factors have been shown to be important for the specificity of the precipitin reaction as revealed by attempts made to standardize the technics of this test. Wolfe (1939) has shown that increasing the amount of antigen injected into rabbits is correlated with a decreased specificity and with greater cross-reactions. It has also been shown that antibodies become more cross-reactive as the period of injection is prolonged (Wells and Osborne, 1913; Wolfe, 1935; Hooker and Boyd, 1941). Another factor which may change the immunologic character of a protein is denaturation. Small degrees of protein denaturation can be produced by such relatively mild treatments as alcohol in the cold (McFarlane, 1935) and drying in the air (Astbury, et al., 1935). Thus it is quite conceivable that acetone drying of pituitary tissue or air drying may result in partial or complete destruction of the specificity of the proteins involved.

The objection to the antibody nature of antigonadotropic substances because of their species aspecificity seems to be without foundation since the methods commonly used in the production of antigonadotropic substances would be expected to yield a serum with a relatively great aspecificity. Some of the factors increasing aspecificity are, *a*) slight denaturation of the proteins used as antigens, *b*) injections of relatively large amounts of protein, and *c*) the prolongation of the period of injection. One or more of these factors may be influencing the specificity of the antigonadotropic substance.

A second objection to the conception of antigonadotropic substances as antibodies is based on the fact that they fail to react as precipitins or complement fixation antibodies. This does not eliminate the possibility that the antigonadotropic substance may be acting similarly to an antitoxin as suggested by Meyer and Wolfe (1939).

Parkes and Rowlands (1937) have suggested that the gonadotropic complex acts as a haptene, and when attached to a protein, the complex will act as an antigen. Similar immunologic reactions are seen in the cases of artificially conjugated proteins (Landsteiner, 1936). Numerous examples are known

which show that antibodies to diazotized proteins are specific for the non-protein haptene and are species aspecific for the protein used to confer the antigenicity to the complex. Most of the data at the present time indicate that the gonadotropic substance is protein or associated with protein. Thus, according to the concept of Parkes and Rowlands, the haptene in question probably is a polypeptide or protein. That such a substance may act as a haptene has been shown by experiments in which an antigen has been made by coupling peptides to proteins. The antibodies produced are very specific for the antigen and the specificity seems to be dependent upon the terminal amino acid of the coupled side-chain (Landsteiner, 1936). The data obtained from the studies of artificially conjugated proteins seem to agree with the theory advanced by Parkes and Rowlands.

Until the gonadotropic substance is more thoroughly investigated and found to disagree with the haptene concept of Parkes and Rowlands, or until the species aspecificity of the antigonadotropic substance is shown to result from factors other than the three mentioned above, a departure from the simple antibody nature of antigonadotropic substances is, at the present time, unwarranted.

CONCLUSIONS

Injections of a horse with extracts of sheep pituitary glands resulted in the formation, first, of a substance which augmented the gonadotropic action of the antigen, then a substance which inhibited the effect of the gonadotropin. The augmenting serum alone failed to produce any demonstrable effects upon the ovaries, vaginae and uteri of normal immature female rats. The serum was found to inhibit the gonadotropins of sheep, rat, beef and human pituitary glands; the gonadotropin from human urine of pregnancy and the serum of pregnant mares was also inhibited. The serum failed to inhibit the gonadotropic activity of horse, hog and chicken pituitary glands.

Preliminary tests, using the oxygen consumption of adult rats as a criterion, indicated that the serum contained no antithyrotropic substance although the antigen was shown to have thyrotropic activity.

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RÔLE OF BILE IN ABSORPTION OF STEROIDS

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BILE PLAYS an important rôle in facilitating the absorption of fat and cholesterol (Schönheimer, 1924) and some of the fat soluble vitamins (Greaves and Schmidt, 1933, 1937; Cohn and Schmidt, 1939) from the gastrointestinal tract. Hence it has generally been assumed that the same would be true of the steroid hormones and their derivatives. In fact bile or bile acids have been used to improve the oral activity of androgens both in experimental animals (Abarbanel, 1940, *a*) and in human patients (Abarbanel, 1939; 1940, *b*, *c*; Silberman *et al.*, 1940). Although Wells *et al.* (1940) showed that in the rat the addition of desoxycholic acid does not enhance the oral effectiveness of testosterone propionate, they admitted that no generalizations can be made from their observations. It is evident that if an excess quantity of bile acids is given to an animal whose liver is already secreting a sufficient amount, an improvement in the absorption rate is not to be expected.

We have recently shown (Selye, 1943) that such steroids as desoxycorticosterone acetate, pregnane-3, 17-dione, testosterone and progesterone are not readily absorbed from the stomach but are rapidly taken up into the blood stream from intestinal segments caudal to the pylorus. The speedy absorption was demonstrated by the fact that they produced anesthesia within 2 hours after their oral administration. Since it is known that a high concentration of steroids must be present in the blood in order to achieve this effect, it is evident that the above compounds are readily taken up by the intestinal epithelium. The question arises whether absorption from the stomach itself is impossible because there is no bile in this part of the gastro-intestinal tract, and whether the presence of bile acids is instrumental in making absorption possible from lower segments of the intestine. It was felt that the crucial experiment for the solution of this problem would be to determine the enteral effectiveness of steroids following ligation of the bile duct.

For this purpose 3 groups, each consisting of 6 female albino rats weighing 28 to 40 gm., were used. In all animals the bile duct was severed between two ligatures. Twenty-four hours later the food supply was withdrawn from the cages so as to empty the gastro-intestinal tract. Forty-eight hours after ligature of the bile duct the animals of the first group received a fine suspension of crystalline desoxycorticosterone acetate in 1 cc. of distilled water administered through a thin stomach tube. The rats of the second group received 10 mg. of progesterone and those of the third group 20 mg. of testosterone in

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the same manner. It should be emphasized that aqueous suspensions of crystalline steroid are much more readily absorbed when given enterally than are solutions in oil given by the same route. Within 45 minutes after such treatment definite signs of drowsiness and loss of equilibrium were demonstrable in the animals of all 3 groups and within 2 hours they were anesthetized. The speed with which the anesthesia developed and the duration of the sleep was approximately identical in these animals. The results were the same in 3 additional groups, each consisting of 6 female rats weighing 30 to 40 gm. and treated in the same manner but without ligation of the bile duct.

From these experiments it may be concluded that the secretion of bile into the intestinal tract plays no important rôle in the enteral absorption of these steroids.

It is noteworthy that methyl testosterone, which has approximately the same anesthetic potency as testosterone when both are given by the parenteral route (Selye, 1942), proved ineffective by mouth even if 30 mg. was given to intact immature females of the same size as those used in experiments reported herein. This was somewhat surprising since it is known that the hormonal potency of methyl testosterone is greater than that of testosterone if both are given *per os*.

It has been assumed that 17-alkyl substituted steroids (*e.g.* methyl testosterone, ethinyl testosterone, ethinyl estradiol, etc.) are especially active by mouth because their absorption from the intestinal tract is better than that of other steroids. The present experimental series is not in agreement with such an interpretation since it shows that compounds, which hormonally are comparatively ineffective by mouth, are rapidly absorbed—as judged by their anesthetic effect—while the reverse is true of the orally effective hormones such as methyl testosterone. It must be kept in mind, however, that for hormonal effectiveness, delayed absorption is preferable to a quick but transient flooding of the organism. Winter (1942) showed that the more rapid the absorption rate, the greater the anesthetic power of steroids, while the reverse is true of their hormonal effectiveness. In the light of these observations it appears more probable that 17-alkyl substitution increases hormonal activity, not because of an improvement in, but because of an inhibition of the rate of intestinal absorption.

It is, perhaps, worth mentioning, in connection with the rôle played by bile in the enteral absorption of steroids, that the effectiveness of long side-chain compounds (*e.g.* cholesterol, stigmasterol) do not necessarily follow the same rules as the hormones which have no, or only a short side chain at C_{17}. Thus it has been found that if compressed pellets of crystalline substances are implanted subcutaneously where they are not exposed to any significant concentration of bile, all of the hormones so far examined are comparatively well absorbed while cholesterol pellets are not absorbed (Forbes, 1942). The question arises whether the great pharmacologic inertia of parenterally administered long side-chain steroids (Selye, 1942) might not merely be due to the inability of the organism to absorb them efficiently.

SUMMARY

Experiments in the female rat indicate that bile plays no important rôle in the absorption from the intestinal tract of steroid hormones. Following oral administration of desoxycorticosterone acetate, progesterone and testosterone a high concentration of these hormones is achieved in the blood so that general anesthesia ensues. This effect is not influenced by complete transection of the common bile duct.

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ADDITIONAL DATA ON THE RELATIVE ABSORPTION RATES OF SUBCUTANEOUS PELLETS OF VARIOUS CRYSTALLINE COMPOUNDS IN THE RAT

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THE DATA here reported are supplementary to earlier observations on the relative absorption rates of pellets of various crystalline compounds in rats. Since the experiments to be reported represent the conclusion of the present phase of a study of pellet absorption, many of the earlier results are briefly recapitulated.

MATERIAL AND METHODS

An apparatus and technique already described (Forbes, 1941) were employed for the preparation of 11 pellets of hexestrol, 26 of diethylstilbestrol dipropionate, 15 of diethylstilbestrol methyl ether (α, α'-diethyl-4-hydroxy-4'-methoxy-stilbene), 12 of diethylstilbestrol methyl ether acetate (α, α'-diethyl-4-acetyl-4'-methoxy-stilbene), 22 of testosterone dipropionate, and 11 of anhydrohydroxyprogesterone.[1] The pellets, made without excipient from the crystalline compound, were uniformly compressed, of standard diameter and, with a few exceptions, weighed 6 to 10 milligrams each.

After careful weighing the pellets were implanted subcutaneously in the lower right abdominal quadrants of 76 male and 23 female healthy adult albino or dark grey-brown rats.[2]

After varying time intervals the animals were killed, and the pellets were removed at autopsy and were dried and reweighed. The percentage loss in weight was then calculated and plotted against the number of days the pellet had been in the animal. An absorption curve was thus derived for each of the 6 compounds. The basis of comparison for a given substance was the number of days which were required for 90 per cent absorption of pellets of the substance.

Each rat was weighed when the pellet was implanted and reweighed at autopsy, and the change in body weight was noted.

The technique for preparation, weighing, implantation, recovery and reweighing of the pellets used in this and in previous experiments was uni-

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[1] These compounds were made available through the generosity of Dr. Ernst Oppenheimer of Ciba Pharmaceutical Products, Inc., Summit, N. J., Dr. Franz C. Schmelkes, Wallace & Tiernan Products, Inc., Belleville, N. J., and Dr. John M. Shaul, Winthrop Chemical Co., Inc., New York, N. Y.

[2] The author is indebted to the Edward Martin Biological Laboratory of Swarthmore College for kindly providing and maintaining the rats used in some of this work.

form. The only significant known variable in the experiments was the compound used.

RESULTS

The absorption curves derived are similar in shape to those obtained in earlier studies (Forbes, 1941; 1942, *b*, *c*) and hence are not reproduced here. The absorption curve for anhydrohydroxyprogesterone is incomplete, since pellets of this compound left in rats for more than 300 days still showed less than 30 per cent absorption. Ninety per cent absorption of the other compounds required the following periods: hexestrol, 54 days; diethylstilbestrol dipropionate, 140 days; diethylstilbestrol methyl ether, 145 days; diethylstilbestrol methyl ether acetate, 220 days; testosterone dipropionate, 272 days.

TABLE 1. TIME REQUIRED FOR 90 PER CENT ABSORPTION OF CRYSTALLINE COMPOUNDS IN PELLET IMPLANTS IN THE RAT

SUBSTANCE	DAYS
Desoxycorticosterone	27
Testosterone	31
Methyl testosterone	36
Diethylstilbestrol	51
Hexestrol	54
Testosterone monopropionate	61
Desoxycorticosterone acetate	64
Progesterone	88
Estriol	93
Androsterone	120
Diethystilbestrol dipropionate	140
Diethylstilbestrol methyl ether	145
α-Estradiol	180
α-Estradiol dipropionate	220
Diethylstilbestrol methyl ether acetate	220
Testosterone dipropionate	272
Estrone (only rough estimate possible)	400
α-Estradiol benzoate	415
Anhydrohydroxyprogesterone	< 30%, 300
Cholesterol	not absorbed

Testosterone dipropionate and diethylstilbestrol dipropionate were the two compounds implanted in both albino and dark grey-brown rats. The two types of animals showed no significant difference in the rate at which they absorbed each of these substances.

At autopsy most of the animals carrying the hexestrol and the 3 varieties of diethylstilbestrol pellets were found to have lost body weight to a significant although highly variable extent (16 to 145 gm. per animal). The loss in body weight associated with a given compound was not necessarily in proportion to the weight of the pellet or to the number of days the latter had been *in situ*.

The alopecia and changes in hair pigmentation caused by some of the compounds have been described in an earlier report (Forbes, 1942, *a*).

DISCUSSION

The absorption rates of the various compounds studied in these and earlier experiments (Forbes, 1941; 1942, *b*, *c*) may now be tabulated for comparison. Unless otherwise specified, the time periods are those required for 90 per cent absorption of the pellets.

For reasons which have already been explained, it is believed that the above time periods are directly comparable and constitute an approximate index to the relative absorption rates of pellets of these compounds. Such an index may be of some value to others in estimating the absorption rates of pellets. The index is also, presumably, an indication of the relative *in vivo* solubility of these compounds in the tissue fluid of the rat. Parallel *in vitro* studies would be interesting. It should be emphasized, however, that one instance has already occurred (Forbes, 1942, *b*) in which a result could not be duplicated within reasonable limits; it is evident that one or more unrecognized variable factors remain to be detected and brought under control before an index of this sort can be accepted without reservation.

SUMMARY

Uniformly prepared pellets, subcutaneously implanted in 99 adult rats, required the following periods for 90 per cent absorption: hexestrol pellets, 54 days; diethylstilbestrol dipropionate, 140 days; diethylstilbestrol methyl ether, 145 days; diethylstilbestrol methyl ether acetate, 220 days; testosterone dipropionate, 272 days. Similarly prepared pellets of anhydrohydroxyprogesterone were less than 30 per cent absorbed in 300 days.

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FURTHER STUDIES ON THE RELATIONSHIP OF PROGESTERONE TO OVULATION AND LUTEINIZATION IN THE PERSISTENT-ESTROUS RAT

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THE PRESENT INVESTIGATION supplements an earlier one (Everett, 1940, *a*, 1940, *b*) in which persistent-estrous rats (Everett, 1939) were temporarily restored to regular ovulatory cycles by the daily injection of small amounts of the corpus luteum hormone. In those experiments certain evidence indicated that progesterone might be required, principally in late diestrus or proestrus. Once persistent estrus had been interrupted by the hormone, the next set of follicles would persist and fail to ovulate unless the daily injections of corpus luteum hormone were continued in the early stages of the new estrus. Several authors (Dempsey et al., 1936; Astwood, 1939; Boling et al., 1940; Boling and Blandau, 1939) have recently shown evidence that progesterone is formed in some degree *before* ovulation in the normal animal. It thus seemed probable that the progesterone, received by the persistent-estrous rats, was substituting for corpus luteum hormone which those animals failed to produce in the presumptive 'preovulatory' period. These considerations led directly to an inquiry as to whether single doses of 0.5 to 1 mg. of progesterone would produce ovulation (luteinization) when given on the first day of estrus.

This proved to be true, as shown by data below. Three other questions immediately arose: *a*) Whether there is a difference in the comparative effectiveness of such single doses when given at different times during the diestrous interval. One might expect an increasing effect toward its close. *b*) Whether a large dose of 2 mg. on the first day of estrus would be as effective as 0.5 mg. It is known from the work of Phillips (1937) that 2 mg. given every every day will suppress ovulation, while 1 mg. or less will not. *c*) For what length of time after a new period of persistent estrus begins could an interrupting dose produce ovulation of the current set of follicles? After 10 days or more, such treatment typically produces atresia of the large follicles (as described later on). They may, nevertheless, be ovulated after equally prolonged estrus by one injection of luteinizing hormone (Marvin and Meyer, 1941).

PROCEDURE

Animals of the DA strain were selected as before (Everett, 1940, *b*), on the basis of their long histories[1] of persistent estrus. In nearly all instances

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[1] A vaginal smear of each animal was made every morning in the manner previously described (Everett, 1939; 1940, *b*).

estrus was interrupted artificially at the start of each experiment by means of a single dose of progesterone,[2] typically 1 mg. This first injection will be referred to henceforth as the *interrupting* dose. In a few instances an interrupting dose was unnecessary because advantage was taken of fortuitous, spontaneous interruptions. With the animal in diestrus, a subsequent experimental (or *test*) treatment was given on one or another specific day of the interval or of the following estrus. Often one animal could be used for more than one experiment; if, for instance, she received 0.5 mg. of progesterone on the second day of diestrus and, nevertheless, returned to persistent estrus, she could be given another comparable dose after she had been in 'heat' for several days.

It was known (Everett, 1940, *b*) that the ovaries of animals with long histories of persistent estrus, such as those which were used for these experiments, possess no luteal elements which might be confused with experimentally induced corpora lutea. The experiments were so designed that usually no more than two sets and never more than three sets of luteal bodies would be formed during the experimental period. These could readily be distinguished from each other histologically. It must be admitted that this was directly a test for luteinization and not for ovulation. However, ovulation was proven by the presence of tubal eggs in 18 of 20 cases in which luteinization had occurred within 48 hours of the time of preservation. Thus formation of corpora lutea of normal appearance may be regarded as evidence of probable ovulation, in the absence of direct proof.

All of the animals were examined at laparotomy at some appropriate time following the final injection, when the vaginal smear indicated *a*) that a cyclic estrus had been completed, *b*) that the animal was returning to persistent estrus, or *c*) that a desired interruption of persistent estrus had been accomplished. The ovaries were exposed for observation and then were quickly removed from the living animal for preservation in Zenker's fluid. As least one ovary from each animal was sectioned at 10μ and every tenth section was mounted. The routine stain was Harris' hematoxylin followed by a modification of Mallory's tri-acid stain.[3] In several cases in which corpora lutea were clearly absent and in a few instances in which there was no question about the character of the corpora lutea (as when rupture points were clearly visible), the ovaries were not preserved.

EXPERIMENTS AND RESULTS

Sources of error. In order to evaluate the effects of experimental treatment after the interruption of persistent estrus, it was necessary to know the likelihood of spontaneous events occurring in spite of experimental treatment rather than as a result of it. Two questions are involved here: *a*) Whether any considerable numbers of animals would, after the interrupting dose of

[2] The progesterone solution was prepared by dissolving the crystals in sesame oil to a concentration of 5 mg. per cc. It was administered by subcutaneous injection in the groin or axilla. Crystalline progesterone was most generously furnished by Dr. Erwin Schwenk of the Schering Corp., Bloomfield, N. J.

[3] The modification is simply that the acid fuchsin is diluted and acidified with acetic acid. Masson's acid fuchsin diluted by 3 parts or more of distilled water is satisfactory.

progesterone, withour further treatment, exhibit ovulatory cycles instead of returning to the persistent-estrous state following the induced interval. *b*) Since persistent estrus is not an absolutely *constant* estrus but on rare occasions may be interspersed with short diestrous intervals (Everett, 1939), the degree of frequency with which spontaneous interruption might be expected to coincide with an attempted experimental interruption.

The first question was answered directly by experiments in which, after the interruption of persistent estrus, further treatment if any, was postponed until it was clearly evident whether or not the new period of 'heat' would persist. Among 45 such cases, there are 2 instances (4.4%) of cyclic estrus with corpus luteum formation. The frequency of such spontaneous cycles would undoubtedly be far higher were the animals not selected on the basis of histories of persistent vaginal cornification for many weeks preceding experimental treatment. In this sense, then, a value of 5% may be regarded as the approximate measure of error in choosing animals for this work.

In attempting to estimate the chances of spontaneous interruption of estrus coincident with experimental treatment, data may be employed from 223 attempts at experimental interruption. Since in 212 (95%) of these the vaginal smear was still largely cornified 24 hours after the injection, becoming diestrous at 48 hours, it may be assumed justifiably that the appearance of a diestrous smear at 24 hours would indicate that estrogen secretion had already been or was about to be interrupted spontaneously at the time of treatment. Of 120 cases, in which the interrupting dose was administered after the 12th day of 'heat,' 3 (2.5%) were found to be diestrous within 24 hours. In 103 cases which received the injection between the 3rd and 12th days of estrus, inclusively, 5 (5%) were diestrous 1 day earlier than expected.

The nature of the experiments is such that these two sources of error are not additive. It may be estimated with reasonable assurance that 90 to 95 per cent of the animals chosen for experiment would, after interruption of persistent estrus, return to that condition following a brief interval and remain so until subsequent treatment might be given.

Induction of cycles. In 125 experiments various test doses of progesterone, from 0.5 to 2 mg. each, were administered at different stages of diestrus or early estrus in an attempt to find the optimum conditions for simulating the normal cycle. When the data were analyzed the best results were found to follow the injection of 0.5 to 1 mg. of the hormone during proestrus after a diestrous interval of 3 days. The data are summarized in tables 1, A and 1, B.

Generally better results could be expected when the diestrous interval lasted for 3 days rather than for 2 days. Tabulation of the results in the *2-day interval* and *3-day interval* categories shows a significantly larger total effect in the latter, with respect to the induction of luteinization. A small number of animals (less than 10 per cent of the entire number assigned to this and the following series) experienced only 1 day of diestrus. Since an interval of such length is decidedly aberrant in terms of the normal cycle, it may be supposed that the interrupting procedure was inadequate. Thus, it may not be sur-

prising that ovulation consistently failed to occur in 10 cases which had been treated during or following a 1-day interval.

An examination of table 1 will show that the later the treatment was given, the greater the number of induced ovulatory cycles. The maximum was reached in those instances when 0.5 to 1.0 mg. of progesterone was injected on the first day of estrus. The results for successful corpus luteum formation for the last 2 days of diestrus in the *3-day interval* experiments and for the first day of estrus in both the *2-day* and the *3-day* groups are all of similar magnitude, between 59 and 79 per cent. However, the upward trend in both groups would seem to be significant. In fact, if the 3 animals which showed a *Stage II* smear at the time of treatment are eliminated from the

TABLE 1. RELATIVE EFFECTIVENESS OF SINGLE DOSES OF PROGESTERONE WHEN GIVEN ON DIFFERENT DAYS OF DIESTRUS OR ON THE FIRST DAY OF ESTRUS

DAY OF ADMINISTRATION OF TEST DOSE	DOSAGE LEVEL, MG.	NO. OF CASES	INTERVAL FOLLOWED BY CYCLE		CORPUS LUTEUM FORMATION	
			NO.	%	NO. CASES	%
A. 2-day interval						
Diestrus, *day 1*	0.5–1.0	1	1		1	
Diestrus, *day 2*	0.5–1.0	15	5	33	5	33
Estrus, *day 1 (stages I, II)*	0.5–1.0	22	22	100	13	59
	2.0	8	8	100	2	25
Totals		46	36	78	21	46
B. 3-day interval						
Diestrus, *day 1*	0.5–1.0	10	2	20	2	20
Diestrus, *day 2*	0.5–1.0	13	9	64	9	64
Diestrus, *day 3*	0.5–1.0	15	11	73	10	67
Estrus, *day 1 (stages I, II)*	0.5–1.0	24	24	100	19	79
	2.0	17	17	100	12	71
Totals		79	63	80	52	66

group treated in estrus, thus restricting the data to individuals treated during proestrus, the value rises from 79 to 90 per cent, with only 2 failures among 21 experiments.

The relative effect of the larger dose of 2 mg. is difficult to evaluate. When all such cases in tables 1, A and 1, B are considered together, the percentage of success is rather low (56%). But if attention is confined to the *3-day interval* experiments, the percentage of positive results (71%) is not unlike that found for the lower dosage levels.

When treatments during diestrus failed to induce corpora lutea there was a prompt return to persistent estrus in all cases save one. On the other hand, treatment on the first day of estrus always caused the interruption of estrous smears within 48 hours, thus giving an outward suggestion of the normal cycle, whether or not luteinization had occurred meanwhile. These facts indicate that progesterone injected during diestrus has a somewhat different

action from that of progesterone injected at the onset of estrus. In the first instance, the effect is registered (if at all) several days later, while in the latter case the effect is much more immediate. To state it in another way: 1 mg. of progesterone given in late diestrus cannot prevent the surge of ovarian secretion which is expected within 48 hours, but once this has occurred, a like quantity of progesterone will readily interrupt it. Afew experiments in which 2 mg. of the hormone was given on the 3rd day of diestrus show that this larger amount will produce a temporary suppression of vaginal cornification 48 hours later, but when luteinization fails, the smear is fully cornified at 72

TABLE 2. RELATIVE EFFECTIVENESS OF SINGLE DOSES OF PROGESTERONE (0.5 TO 1.0 MG.) AS PERSISTENT ESTRUS ADVANCES

DAY OF ESTRUS	NUMBER OF CASES	CORPUS LUTEUM FORMATION	
		NUMBER OF CASES	%
1[1]	46	32	70
2–3 inclusive	19	7	37
4–5 inclusive	19	5	26
6–7 inclusive	19	3	16
8–9 inclusive	17	2	12
10–100 inclusive	79	8	10

[1] Combined data from tables 1A and 1B

hours and continues so. The temporary suppression of cornification at 48 hours is interpreted here as the result of direct but fleeting action of progesterone on the vagina.

Changes in ovarian histology as a result of treatment were rather clear-cut in most instances. As indicated above, the method of selection of animals precluded the existence of luteal elements which might be confused with those experimentally induced. The ovary in persistent-estrous rats characteristically contains a set of large follicles (diameter ca. 1 mm.), 'cystic,' perhaps, but capable of being ovulated and luteinized when given adequate stimulus (Everett, 1940, *b*; Marvin and Meyer, 1941). The interstitial cells exhibit 'wheel' nuclei indicating a degree of hypophyseal deficiency. This condition is most marked where the cells lie at some distance from the large follicles. In the present experiments these follicles usually became atretic as the result of the interrupting dose of progesterone. A new set then grew during the diestrous interval. When progesterone was given during this interval, and yet failed to produce corpora lutea, the new follicles persisted, possibly being replaced from time to time by younger ones. The interstitial cells continued to show their deficiency reaction. If, however, ovulation was accomplished, corpora lutea formed which were indistinguishable from those in the normal rat. When such luteinization occurred, it was accompanied by extensive repair of the interstitial cells, especially in the lobes containing the corpora lutea and in the deeper parts of the other lobes (Everett, 1941). The degree of this repair seemed to be directly correlated with improvement of circulation and associated increase of tissue fluid.

Similarly, in the experiments in which progesterone was injected during early estrus, successful cases have shown new corpora lutea and marked interstitial-cell repair 48 hours later. In the instances in which ovulation failed, the large follicles were usually undergoing cystic atresia at such time, although in one animal several of the atretic cysts showed patches of theca lutein cells in their walls. Failure of full luteinization was accompanied by apparent absence of interstitial-cell repair.

Luteinization accompanying interruption of early persistent estrus. In 89 experiments a test injection of progesterone (0.5 to 1.0 mg.) was given on one of the early days of persistent estrus, in the period from the 2nd to the 12th day, inclusively. This treatment regularly produced a diestrous smear within 48 hours and, in 17 instances, interruption of cornification was accompanied by corpus luteum formation, again with interstitial-cell repair. In the others the large follicles merely became atretic. In 12 of the 17 ovulation followed injection on the 2nd to the 5th days, inclusively, as indicated in table 2. Possibly the combined results indicated for the 2nd and 3rd days of estrus would have been higher had the data for the 2nd day not been derived entirely from cases in which the preceding interval had lasted only 2 days, since they were all negative. The *2-day interval* cases showed considerable refractoriness to progesterone, as described above.

In general, it seems that after a peak of luteinization in 60 or 80 per cent of the cases following progesterone treatment on the 1st day of estrus there is a gradual downward trend through the following 7 to 8 days. Thereafter the injection of progesterone will nearly always result in cystic atresia of the large follicles. As it happened, 20 cases treated on the 9th, 10th, 11th or 12th days of estrus were all anovulatory. Occasionally, however, luteinization, and presumably ovulation, may accompany the experimental interruption of persistent estrus of even longer duration. Definite information on this question is furnished by data from 79 animals in which estrous smears had been exhibited continuously for 10 to 100 days, 64 for periods longer than 20 days. Eight (10%) of the 79 animals formed full sets of corpora lutea directly following the interrupting dose.

DISCUSSION

After long-continued persistent estrus, a single (interrupting) injection of progesterone (0.5 to 1.0 mg.) typically will cause cessation of vaginal cornification, accompanied by precipitous atresia of the large follicles. The smear becomes diestrous within 48 hours and usually remains so for 2 or 3 days. A second (test) injection of progesterone late in the interval will usually be followed by an 'ovulatory' cycle. Without the second injection the animal returns to persistent estrus and the current follicles remain unruptured. Outwardly normal cycles are always observed when the test injection is given on the 1st day of vaginal estrus; 60 to 80 per cent of these cycles are 'ovulatory.' When estrus is allowed to continue for a short time before giving the test injection, the frequency of ovulation gradually diminishes through

the first 8 days. Thereafter ovulation may be expected in only 10 per cent of the cases.

Progesterone is thus most effective when it operates at a time comparable to that of preovulatory swelling of the graafian follicles. In the original experiments with daily administration of the hormone (Everett, 1940, *a*, *b*), it may be supposed that it was made available at a sufficient level whenever the animal required it. A re-examination of the ovaries from the series receiving daily injections has given clear evidence that luteinization had occurred in 86 per cent of 22 cycles. This value is not unlike the best results obtained in the present series, namely, with one injection during proestrus after a 3-day interval.

The observations recorded here tend to supplement evidence (Dempsey, et al., 1936; Astwood, 1939; Boling et al., 1940; Boling and Blandau, 1939) which has indicated that in the normal animal progesterone is formed to some extent before ovulation occurs. In this connection it is noteworthy that, in *Elephantulus*, luteinization of the follicles begins before their rupture (van der Horst and Gillman, 1942) and is apparently an essential step in the ovulatory process. One is also reminded of certain observations made by Boling, Barton and Burr (1940) in studies of the electrical potential changes in the vagina associated with the estrous cycle. Evidence was presented which indicated that the electrical fluctuations characteristic of ovulation may be produced experimentally not by estrogen alone, but by estrogen followed by progesterone. Two instances were then described of rats which failed to show the ovulation E.M.F. pattern when expected. Their ovaries, when removed later and studied histologically, showed 'large unruptured follicles with no new corpora lutea.' It is possible that these were instances of incipient persistent estrus.

The evidence accumulated to date suggests that, in the rat, progesterone secretion in the preovulatory phase of the cycle is either an essential step in the sequence of events leading to ovulation, or a safety factor which tends to insure a balance of gonadotropic hormones which is compatible with ovulation. The inference exists that, in the persistent-estrous rat, progesterone fails to be produced at the proper time. When the hormone is made available by correctly timed injection, the full morphologic pattern of the normal cycle is elicited. On the basis of additional evidence which cannot be presented here it seems probable that progesterone is not an absolute prerequisite to the completion of this pattern, but is, rather, a safety factor. Its mode of action must remain in question, although there seems to be good reason to think that at a certain stage of the pituitary cycle in the rat (i.e. in the period when the animal is just entering estrus) progesterone momentarily facilitates release of the luteinizing hormone although, when present continuously in large amounts, the corpus luteum hormone is thought to suppress the output of LH in the rat (Astwood and Fevold, 1939) as well as in other animals (Makepeace, et al., 1937; Dempsey, 1937). No explanation can be offered for the considerable impetus to ovulation which followed the single injections at the midpoints of *3-day diestrous* intervals.

SUMMARY

Persistent estrus in the DA strain of rats was interrupted at will by a single injection of 0.5 to 1 mg. of progesterone, usually causing atresia of the large follicles and the appearance of a diestrous smear, lasting 2 or 3 days as new follicles were growing.

A second injection was given on specific days of this diestrus or of the following estrous period. The effectiveness of such treatment in producing corpus luteum formation was slight in early diestrus, rose to a maximum on the 1st day of estrus (*stages I* or *II*) and then declined gradually to a negligible value when estrus had persisted longer than 8 days.

The evidence tends to support the work of others which has indicated that, in the normal animal, progesterone begins to be secreted in the preovulatory phase of the estrous cycle. The repair of interstitial cells in the ovary which accompanied successful corpus luteum formation indicates that progesterone at certain times may cause a (momentary?) release of pituitary luteinizing hormone, at least in this one species and under these special conditions.

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SELECTIVE BISMUTH MELANOSIS OF THE FEMALE GENITAL TRACT INDUCED BY TREATMENT WITH SEX HORMONES[1]

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WHILE carrying out a pharmacologic study on a number of bismuth preparations, vaginal impregnation with bismuth was observed in one series of rats but in none of the others. Subsequent search into the causes of this phenomenon indicated that bismuth impregnation of the vagina occurs only if the rats are in permanent estrus.

EXPERIMENTAL PROCEDURE

Animals. Experiments with bismuth preparations were made on a total of 182 young female albino rats weighing 60 gm. at the outset and 120 gm. at the end of the treatment 2 months later. A total of 122 animals received bismuth preparations together with estrogenic or gonadotropic hormone administered by injection at different sites in the neck; 60 animals served as controls. In addition, 22 young male albino rats were treated with bismuth preparations and testosterone propionate. Ten male animals served as controls.

Bismuth preparations. Bismuth-phenyl-ethyl-acetate (25% Bi), Bi-butyl-thio-laurate (25% Bi), Bi-chinolinate (45% Bi), Bi-adipinate (66% Bi) were used. The first two were injected in oily solution, the latter two, being insoluble in oil, as suspensions. In each case the daily dose consisted of the equivalent of 3.5 mg. of Bi-metal.

Sex hormone preparations. Stilbestrol dipropionate,[2] estrone[3] and estradiol benzoate[3] were injected in oil, chorionic gonadotropin[3] in aqueous solution were used. These preparations were administered (see table 1) simultaneously with Bi compounds but at different sites.

RESULTS

A total of 122 female rats was injected with various bismuth preparations at a rate of 3.5 mg. of metallic bismuth daily. Those animals receiving estrogenic hormone or chorionic gonadotropin concomitantly with bismuth reacted with strong bismuth impregnation of the genital tract, especially of the

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[2] These compounds were generously supplied by the Teva, Middle East Pharmaceutical and Chemical Works Co. Ltd., Jerusalem.
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vagina. The impregnation was not demonstrable in 60 control rats which received the same bismuth treatment without sex hormones. Non-genital organs (*e.g.* gingiva) were never found impregnated in either group. Details are given in table 1.

It should be stressed that impregnation of the vagina is not obtained in all of the animals treated, the proportion ranging between 30 and 80 per cent. The factor which rules the incidence of the impregnation is difficult to determine with certainty. The amount of estrogenic hormone given does not

TABLE 1. EFFECT OF SIMULTANEOUS BISMUTH AND SEX HORMONE TREATMENT ON BISMUTH MELANOSIS IN THE VAGINA OF YOUNG FEMALE ALBINO RATS

NUMBER OF RATS	HORMONES, AMOUNT GIVEN DAILY, MG.	ANIMALS SHOWING BISMUTH MELANOSIS OF THE VAGINA		EARLIEST DATE OF REACTION	LATEST DATE OF REACTION	NUMBER OF ANIMALS REACTING WITHIN ONE WEEK OF TREATMENT	
		NO.	%	DAY	DAY	NO.	%
		3.5 mg. Bi daily as Bismuth phenylethylacetate, and estrogen[1]					
20	1.00	10	50	7	34	4	20
10	1.00	5	50	6	17	2	20
4	1.00	2	50	28	28	0	0
10	0.50	8	80	6	39	5	50
10	0.01	2	20	7	16	1	10
10	1.0[2]	3	30	7	51	2	20
8	0.5[2]	3	37	52	56	0	0
10	2 I.U.[3]	6	60	6	48	5	50
10	0.03[4]	5	50	7	24	2	20
		3.5 mg. Bi daily as Bismuth butylthiolaurate, and estrogen					
10	0.5	7	70	7	85	3	33
		3.5 mg. Bi daily as Bismuthchinolinate, and estrogen					
10	0.01[4]	7	70	6	31	1	10
		3.5 mg. Bi daily as Bismuthadipinate, and estrogen					
10	0.01[4]	0	0	0	0	0	0
		3.5 mg. Bi daily as Bismuth phenyl ethyl acetate, no estrogen					
60	0.0	0	0	0	0	0	0

[1] Estrogen as diethylstilbestrol dipropionate was used unless indicated otherwise.
[2] Estradiol benzoate.
[3] Chorionic gonadotropin.
[4] Estrone.

appear to be decisive, since we obtained the same results with doses as high as 1 mg. of diethylstilbestrol dipropionate daily and with doses as low as 0.03 mg. of estrone. Working with chorionic gonadotropin, we avoided high luteinizing dosages in order to achieve a preponderant estrogenic effect. In this case we obtained impregnation of the vagina even with 2 I.U. daily.

The last column of table 1 throws some light on the question of the possible significance of dosage. It may be seen that early vaginal impregnation was obtained in 50 per cent of the rats receiving 2 I.U. of prolan and 0.5 mg. of diethylstilbestrol dipropionate daily. This confirms the assumption that a high dosage of sex hormones is not necessary in order to precipitate the impregnation reaction.

On the other hand, it appeared that the nature of the bismuth compound used has an important bearing on the impregnation reaction, bismuth adi-

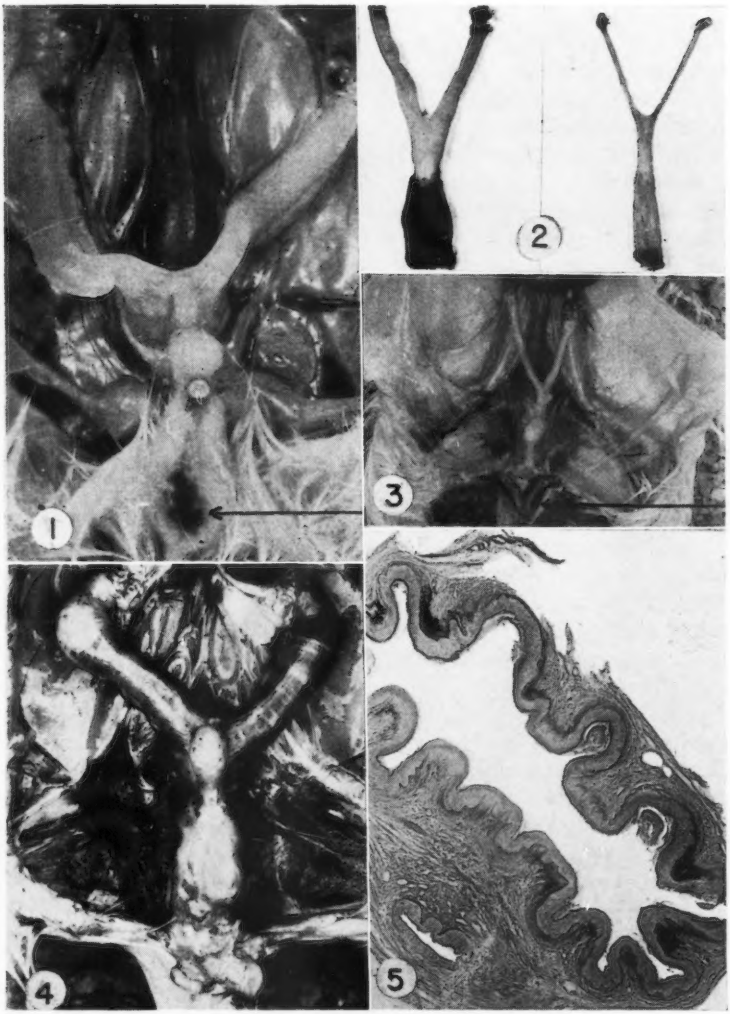

Fig. 1. Black introitus vagina in rat treated with bismuth preparation and estrogenic hormone; typical example.

Fig. 2. (*left*) Blackening of the whole vagina in rat treated with bismuth preparation and estrogenic hormone; extreme example. (*right*) Control specimen.

Fig. 3. Blackening of the preputial gland in rat treated with bismuth preparation and estrogen hormone; rare picture.

Fig. 4. Blackening of the uterine horns in rat treated with bismuth preparation and estrogenic hormone for over two months. Suppuration of the uterus.

Fig. 5. Histologic picture of bismuth melanosis of the vagina in rat; brief fixation in formalin, frozen section stained with fuchsin. Note the impregnation zone along the lamina propria mucosae. ×250.

pinate proving inactive, as compared with the other 3 test compounds. That this inactivity is not related to the solubility of the bismuth compound, nor to the presence therein of a phenol group, is proved by the activity of the 3 other bismuth compounds tested. The cause of inactivity of the bismuth-adipinate therefore remains an open question.

In the experiments reported in table 1 and in all our considerations of the impregnation reaction, blackening of the vagina served as the sole criterion. It should be mentioned, however, that sometimes also other reactions may occur. Normally the blackening of the vagina is easily recognizable in the living animal by inspection of the anterior wall of the vagina, the posterior wall appearing a metallic black against the feces contained in the rectum. The typical picture is shown in figure 1. Sometimes the blackening of the vagina is so outstanding that a distinct differentiation between the lower urogenital segment and the higher uterine or muellerian duct segment is possible. A typical picture of this condition is given in figure 2.

In extraordinary cases, bismuth melanosis occurs at sites other than the vagina, especially if high dosages of estrogens are used. In one case we observed a blackening of the preputial glands (fig. 3).

If treatment with estrogen together with bismuth injections in high dosage is protracted for more than 2 months destructive changes of the uterus may result as described in 1937 by Zondek in rats treated with estrogenic hormone. In the animal pictured in figure 4 the uterine horns were thickened to pencil-sized structures full of pus; small black segments indicated the bismuth impregnation, the vagina itself being only insignificantly impregnated. This rat had been treated for 70 days with 1 mg. of estradiol benzoate and 5 mg. of bismuth-butyl-thio-laurate daily. Changes such as those shown in figure 3 and 4 are not, however, the general rule.

In general, the histologic appearance of the vagina in bismuth melanosis is distinctive: a subepithelial layer of the mucosa is colored brown by the bismuth, especially in the papillae (fig. 5), the substance being restricted to the connective tissue only. In histologic preparation prolonged fixation in formol and paraffin imbedding had to be avoided, since these procedures caused a loss of the color. The best preparations were obtained by boiling for a few minutes in 4 per cent formol (1:10) sectioning with the freezing microtome, followed by staining with a red dye such as eosin or fuchsin. Bismuth impregnation of the vagina could be verified also by the deepened shadow of the vagina in roentgen-ray photographs. This last test did not, however, prove consistently reliable, the amounts of bismuth in the vagina being too small, frequently, for detection by roentgen-ray.

Attempts to obtain a similar local reaction to bismuth compounds in male rats were made with testosterone propionate. The male genital tract of these animals did not, however, show specific pigmentation, except for occasional blackening of the preputial glands.

SUMMARY

Female albino rats simultaneously treated with organic bismuth compounds and estrogens or chorionic gonadotropin developed bismuth melanosis

of the vagina in 30 to 80 per cent of the cases. The reaction was conditioned primarily by hyperemia of the vagina under the influence of the hormones used and secondarily by an additional unknown factor which is responsible for the fixation of bismuth selectively in the vagina. Bismuth melanosis in sexual organs other than the vagina, *e.g.* the preputial glands or the uterus, was seldom observed, the rest of the body was always found free of bismuth impregnation.

In male rats simultaneously treated with bismuth and testosterone propionate, bismuth impregnation of the genital tract occurred only occasionally.

The value of these findings for chemotherapy is suggested.

We are greatly indebted to Prof. B. Zondek, head of the laboratory, under whose supervision this work was done.

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RELATION OF CERTAIN ENDOCRINE GLANDS TO BODY WEIGHT IN GROWING AND MATURE GUINEA PIGS[1,2]

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IN ENDOCRINE studies involving the use of experimental animals, it is important to be able to differentiate between the normal weight changes of the several endocrine glands associated with increasing body weight and changes which may be due to experimental conditions. Even when a considerable number of control animals are included in an experiment, it is difficult to determine the influence of the experimental condition upon gland weight, if the experimental treatment also influences growth rate, unless controls of various body weights are autopsied. Under such conditions, standards of normal endocrine gland weight with increasing body weight should be valuable reference data for comparative purposes.

Since gland weights in general do not vary directly (to the first power) with body weight, but to some other power of body weight, the power formula or the relative growth equation $Y = aX^b$ has been used widely in statistical studies relating organ weight to body weight (Du Bois, 1898; Lapicque, 1898; Huxley, 1932; Quiring, 1938; Brody and Kibler, 1941).

In the course of several experiments in our laboratory many data have been collected on the normal weights of the pituitary, thyroid, adrenal, gonad and body of both the male and female guinea pig. In this paper the pituitary, thyroid, adrenal and gonad weights of both the male and female guinea pig were related to body weight using the relative growth equation as mentioned above. It is hoped that these data may serve as useful standards of reference for endocrine gland weights for the male and female guinea pig.

EXPERIMENTAL PROCEDURE

The guinea pigs used in this study were fed a complete grain mixture to which was added Cerogras[3] to the amount of 10 per cent of the mix. This was supplemented by fresh greens such as lettuce and carrot tops. The animals were kept at an environmental temperature of about 80°F. and they were killed during the fall, winter and early spring months. None of the animals were used for breeding purposes.

The animals were killed with ether, body weights were recorded to the

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nearest gm. and the whole pituitary, the thyroid, the paired adrenals and the paired gonads were weighed to the nearest 0.1 mg.

Separate statistical analyses were made on the data from the male and female animals. To judge the trend of the data, the body weights were grouped into 50-gm. class intervals and for these class intervals average body and gland weights were determined. These average figures for gland and body weight gave fairly straight lines when they were plotted against each other on double logarithmic paper. The relative growth equation $Y = aX^b$ was fitted

TABLE 1. MEAN BODY AND GLAND WEIGHTS OF 207 GROWING MALE GUINEA PIGS GROUPED INTO 50-GRAM BODY WEIGHT GROUPS

NUMBER OF ANIMALS	AVERAGE				
	BODY WEIGHT	PITUITARY WEIGHT	THYROID WEIGHT	ADRENAL WEIGHT	GONAD WEIGHT
	gm.	mg.	mg.	mg.	mg.
13	131.5	5.3	23.6	66.7	97.0
10	176.7	6.5	23.3	86.3	194.0
18	224.6	9.2	32.5	112.4	346.3
15	280.2	10.5	37.5	144.5	706.9
26	317.0	10.9	39.8	150.0	845.0
10	382.2	13.6	45.0	188.7	1447.0
23	420.2	14.5	53.6	222.0	1707.8
16	486.2	15.1	59.0	247.4	2508.6
14	523.6	15.8	65.6	334.6	3000.3
14	575.1	16.4	58.5	392.6	3439.2
15	621.0	17.2	62.2	392.7	3732.1
10	672.1	18.6	61.9	442.0	4138.2
9	721.0	17.4	65.4	486.9	4370.5
5	762.1	18.3	90.5	626.6	4517.5
5	834.8	20.1	102.0	586.9	4226.2
1	885.0	19.4	91.0	533.8	2949.0
1	920.0	25.9	110.0	628.5	4656.0
2	1012.5	22.8	127.5	549.3	4899.8

to the individual data points by the method of least squares, Y being gland weight and X being body weight. The standard errors of estimate of the regression equations were calculated and are shown in the figures.[4] The lines of the standard error of estimate include between them $\frac{2}{3}$ of all the original data points from which the equation was calculated. The index of correlation, represented by the letter *rho*, was also calculated for each group of data and it is likewise shown in the figures. This corresponds to the coefficient of correlation of linear equations.

Analysis of Data

Pituitary and body weight. In this study of the relation between pituitary and body weights 207 male and 146 female guinea pigs were included. Separate equations for the male and the female were fitted to the individual data

[4] Tables were prepared showing *a*) the calculated endocrine gland weights with increasing body weights, *b*) the ratios of gland weight to body weight as body weight increases, and *c*) percentage increases in gland weight corresponding to various percentage increases in body weight. At the request of the Editors, these tables have been omitted, but they have been mimeographed and will be sent to interested readers on request.

points for pituitary-body weight. These regression lines (numbers 1 and 2) together with their standard errors of estimate are presented in figure 1.

(1) Male $Y(\text{pituitary weight}) = 0.183X^{0.708}$ (body weight)
(2) Female $Y(\text{pituitary weight}) = 0.140X^{0.732}$ (body weight)

These equations may be used to predict pituitary weights of male and female guinea pigs from birth to 1000 gm. of body weight. According to these data,

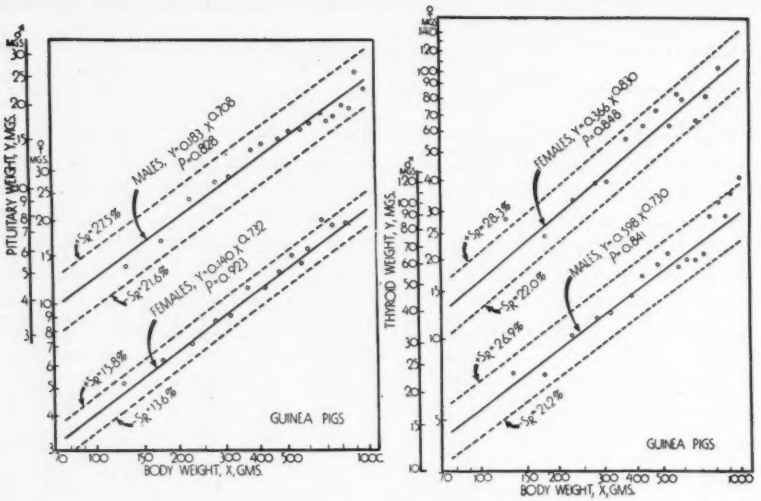

Fig. 1, *left*. Relation of pituitary weight to body weight in growing and mature guinea pigs plotted on a logarithmic grid. In all of the figures the heavy continuous lines represent the equation $Y = aX^b$ fitted by the method of least squares to the individually paired gland-body weight data. The broken lines on both sides of equation lines represent the standard error of estimate, S_r, including between them in each case two-thirds of the data; the letter *rho* represents the index of correlation. The male guinea pig has a heavier pituitary than the female in this species of animal.

Fig. 2, *right*. Relation of thyroid weight to body weight in growing and mature guinea pigs plotted on a logarithmic grid. The thyroid in the female increases in weight at a faster rate during growth of the guinea pig than it does in the male. The thyroid of the mature female is heavier than that of the male.

the male guinea pig has a slightly larger pituitary than the female. These equations indicate that pituitary weights do not vary directly with body weights but vary with the 0.708 and 0.732 power of body weights, respectively, in the male and female guinea pigs. Thus the ratio of pituitary weight to body weight decreases as the body weight increases. An increase of 100 per cent in body weight causes increases of only 63.4 and 66.1 per cent in the pituitary weights of the males and females respectively, while an increase of 1 per cent in body weight causes 0.708 and 0.732 per cent increases, respectively, in pituitary weights, which figures are identical with the values of *exponents b* in the equations. Thus *exponent b* may be interpreted as the relative change in pituitary weight, Y, corresponding to a small unit change in body weight, X.

These *b* values for pituitary and body weight have been determined for a number of other species of animals. Brody and Kibler (1941) have determined that the *b* value for fetal and new-born cats is 0.45, for male albino rats 0.74, for female albino rats 0.93, while for mature animals of different species the *b* value is 0.76. Kibler, Bergman and Turner (1942) have determined the *b* value of male and female New Zealand white rabbits to be 0.58

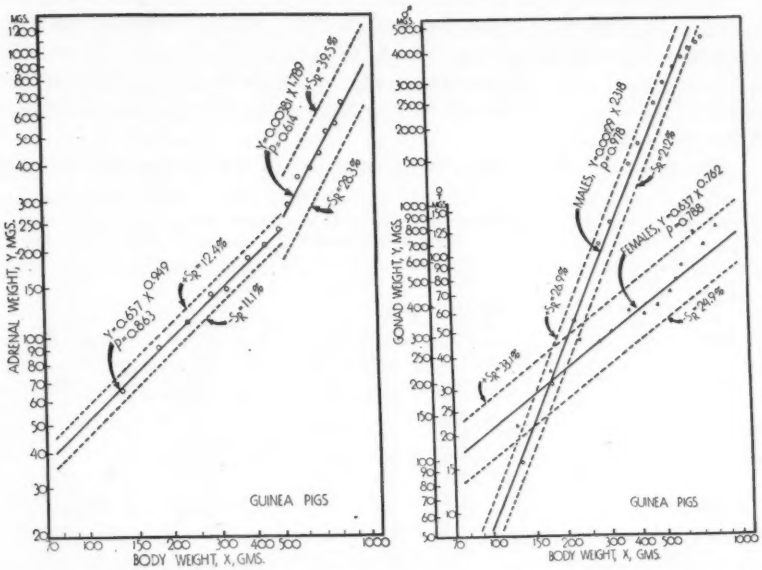

Fig. 3, *left*. Relation of adrenal weight to body weight in growing and mature guinea pigs plotted on a logarithmic grid. The guinea pig displays no difference in adrenal weight due to the sex of the animals. There is a marked increase in the rate of adrenal growth of animals over 500 gm. of body weight as compared to animals under 500 gm. of body weight as noted by the slopes of the regression lines for animals under 500 gm. and those over 500 gm. of body weight.

Fig. 4, *right*. Relation of gonad weight to body weight in growing and mature guinea pigs plotted on a logarithmic grid. There is a very marked difference in the rate of gonad development of the male and female guinea pig with increasing body weight, the testis increasing in weight at a much greater rate than does the ovary with increasing body weight.

and 0.68, respectively, while Mixner and Turner (1942) have shown that for Wistar strain male albino rats, *b* is 0.68.

Thyroid and body weight. Again, as with the pituitary, 207 male and 146 female guinea pigs were used in this study of the relation between thyroid and body weights. Equations for the male and female were fitted to the individual data points for thyroid-body weight. Figure 2 presents these regression lines (numbers 3 and 4) together with their standard errors of estimate.

(3) Male $Y(\text{thyroid weight}) = 0.598X^{0.730}$ (body weight)
(4) Female $Y(\text{thyroid weight}) = 0.366X^{0.830}$ (body weight)

Thyroid weight prediction values as calculated from these formulae indicate that, although the thyroids in the males and females started at about the

same level of weight, in the female the thyroid increased in weight at a greater rate than in the male, making the thyroid of the mature female heavier than that of the male. The equations indicate that, as with the pituitary, the thyroids do not vary directly with body weight but to the 0.730 and 0.830 power of body weight, respectively, for the male and female. Thus, as body weight increases the ratio of thyroid weight to body weight decreases.

The thyroid-body weight *b* values of 0.73 and 0.83 for the male and female guinea pigs, respectively, may be compared to *b* values for other species of animals as determined by Brody and Kibler (1941). The *b* value for growing rats is 0.81, fetal and newborn cats 0.77, growing chickens 1.2, premature and newborn horses 0.95, horses over 1 year old 1.17 and for mature mammals of different species 0.92.

Adrenal and body weight. An examination of the average adrenal-body

TABLE 2.—MEAN BODY AND GLAND WEIGHTS OF 146 GROWING FEMALE GUINEA PIGS GROUPED INTO 50-GRAM BODY WEIGHT GROUPS

NUMBER OF ANIMALS	AVERAGE				
	BODY WEIGHT	PITUITARY WEIGHT	THYROID WEIGHT	ADRENAL WEIGHT	GONAD WEIGHT
	gm.	mg.	mg.	mg.	mg.
8	127.0	5.2	28.1	67.1	21.7
29	176.6	6.3	24.6	97.3	35.3
25	226.7	7.2	33.3	114.1	47.3
24	280.1	8.7	39.2	140.2	46.2
14	317.7	9.1	39.7	146.5	50.0
6	369.5	11.4	56.5	199.2	60.8
6	427.0	11.4	63.9	308.2	60.1
11	477.7	13.0	73.2	233.4	64.3
8	536.0	15.0	63.0	196.5	71.5
5	577.0	14.1	84.1	295.0	80.5
1	602.0	15.8	79.4	393.3	91.0
3	675.7	20.5	67.0	485.2	125.8
2	742.5	19.3	81.5	725.5	110.4
4	837.5	19.7	103.8	627.4	130.4

weight data for the male and female guinea pig as seen in tables 1 and 2 revealed two things. First, there was no discernible difference in the trend of the data for male and female guinea pigs when these points were plotted on logarithmic paper and secondly, there was a distinct break in the slope of the lines of means of data for both the male and the female at the level of 500-gm. body weight class. It was evident that the slope or the *b* value for the equation representing body weights under 500 gm. was under 1, while the slope or *b* value for the equation representing body weights over 500 gm. was considerably over 1. As a result of this examination it was decided to combine the data for males and females and treat them as one population (the sex differences being negligible) and separate regression equations were calculated for the data representing body weights (male and female combined) below 500 gm. and for those above 500 gm.

The data for body weights under 500 gm. included those from 131 male

and 123 female guinea pigs, a total of 254 animals. An equation was fitted to these 254 individual data points for adrenal-body weight and this regression line (number 5) is presented in figure 3, together with its standard error of estimate.

(5) Male and Female (Under 500 gm. body weight) $Y(\text{adrenal weight.}) = 0.657X^{0.949}$ (body weight)

In this equation the *b* value of 0.949 approaches 1 very closely and thus the rate of adrenal development closely follows that of body weight. As body weight increases by 100 per cent, adrenal weight increases by 93 per cent over the body weight range up to 500 gm.

The data for body weights over 500 gm. include that for 72 male and 21 female guinea pigs, a total of 93 animals. The regression equation (number 6) for these data is presented in figure 3 together with its standard error of

(6) Male and female (Over 500 gm. body weight) $Y(\text{adrenal weight}) = 0.00381X^{1.789}$ (body weight)

estimate. The power of X (*b* value) in this equation is > 1, 1.789, and in these guinea pigs at a body weight greater than 500 gm. the adrenals increase in weight at a greater rate than does body weight. Thus, the ratio of adrenal weight to body weight over 500 gm. increases as body weight increases. This is the only case so far in which this has been true. Indeed an increase of 100 per cent in body weight (from 500 to 1,000 gm.) is associated with an increase of 245 per cent in adrenal weight.

The accelerated rate of growth of the adrenal glands in the heavier animals is an interesting observation. Is it due to increased secretion of adrenotropin by the pituitary causing cortical hyperplasia or is the medulla chiefly concerned or responsible for the increase in adrenal weight?

Again, as for the pituitary and the thyroid, the *b* values for adrenal-body weight of 0.949 (under 500 gm. body weight) and 1.789 (over 500 gm. body weight) for guinea pigs may be compared to *b* values for other species as determined by Brody and Kibler (1941). The *b* value for immature dogs is 0.78, growing male rats 0.77, growing female rats 0.77, fetal and newborn cats 0.64, growing chickens 0.84, growing horses 0.71 and for mature mammals of different species 0.798.

Gonad and body weight. Male. In the study of the relation of testis weight to body weight no animals weighing more than 800 gm. were included. Above this point the mean-values of testes weight to body weight became so irregular that they were useless for purposes of determining a regression line. Consequently, only data from 198 of the 207 males were included in this study. An equation was fitted to the individual data points for the gonad-body weight. This regression line (number 7) is presented in figure 4, together with its standard error of estimate.

(7) Male $Y(\text{testis weight}) = 0.00129X^{2.319}$ (body weight)

As may be seen from the formula, testis weight increases at a much greater

rate than does body weight ($b > 1$), varying with the 2.319 power of body weight. As body weight increases 100 per cent, testis weight increases 398 per cent. Also the ratio of testis weight to body weight increases from 0.039 per cent at 75-gm. body weight to 0.866 per cent at 800-gm. body weight, a change of over 20 times in the relative ratios at these two body weights. *Female.* In the study of ovary body weight relations, 146 animals were included. A regression equation was determined relating ovary weight to body weight. This equation (number 8) with its standard error of estimate is presented in figure 4.

(8) Female $Y(\text{ovary weight}) = 0.637X^{0.762}(\text{body weight})$

It may be seen from the equation that ovary weight varies to the 0.76 power of body weight and as b is < 1 the ratio of ovary weight to body weight decreases as body weight increases. This is in contrast to the male in which the testis weight increases at a much greater rate than does body weight.

SUMMARY

Using the relative growth equation $Y = aX^b$, pituitary, thyroid, adrenal and gonad weights of 207 male and 146 female growing guinea pigs were related to their body weights. The equation was fitted to the data by the method of least squares. Equations and graphs for predicting the various endocrine gland weights from body weights are presented. These data are presented with the idea that they may serve as useful standards of reference for endocrine gland weights for the male and female guinea pig.

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NEW BOOKS

THE HORMONES IN HUMAN REPRODUCTION. George W. Corner, Director, Department of Embryology, Carnegie Institution of Washington, Baltimore, Md. Cloth. $2.75. 265 pages; 24 plates, 32 text figures. Princeton, N. J. Princeton University Press. 1942.

This volume is a skillful refutation of the current concept that scientific writing, to be accurate, must also be dull.

These essays represent, with additions, the substance of the Vanuxem Lectures, given at Princeton University in February of 1942. The author's preface states that the lecturer was requested " . . . to discuss the hormones of the reproductive system for the benefit of a general audience, assuming on the part of my hearers no familiarity with biology." How extraordinarily well the author has fulfilled his mission cannot be appreciated until the book has been read. It is not essentially a book for the specialist in this field, although no one will read it with greater profit or keener delight. It tells the story of reproductive physiology with complete accuracy of detail, but with such simplicity of statement and charm of style that those who are not familiar with biology and chemistry will be carried along by the flow of the story as it reveals the steps by which knowledge of the sex hormones was attained, told in the words of a skilled narrator.

The first chapter provides the framework of the general biological pattern of sex and reproduction. The titles of the subsequent chapters are suggestive of the interest to be found in them. 'The ovary as a timepiece' (Chapter III) is a study of periodicity and reproductive rhythms and the estrous cycle. 'Endocrine arithmetic' (Chapter VII) is an amplification of previous work of the author on the rate of secretion of ovarian hormones. The results of these arithmetical manipulations are presented as suggestive inferences of glandular and cellular activity. One computation would indicate that if estimates of progesterone production are anywhere near correct " . . . the amount present in the ovaries (of the sow) at any one time is less than two minutes' supply."

In previous publications, Dr. Corner has made contributions to the history of science, but there has been no single chapter in contemporary writing where the story of the completion of a single scientific achievement is so completely told as in 'A hormone for gestation' (Chapter IV). This is a tale of research, of the steps taken by many men, in revealing the rôle of the corpus luteum, including the crystallization and naming of its hormone, progesterone.

The chapter is highly personalized, and the author recaptures and transmits the enthusiasm which must have been his own during the first attempts to obtain an active fraction from the gland. Credit is given and taken where due, and the individual worker appears in his place as each successive stage of the research leads to another phase. The general reader shares with the investigator "the memories of bafflement, comedy, hard work and modest success." The reader as well as the author will not forget " . . . the time I went racing up the steps of the laboratory in Rochester, carrying a glass syringe that contained the world's entire supply of crude progesterone, stumbled and fell and lost it all."

In discussing matters on which there may not be quite general agreement among his contemporaries, such as the question of post-natal formation of new ova, or the exact weighing of factors involved in menstruation, the author has written carefully and judiciously. It is probable that the general reader may find the discussion of experimental evidence on the hormonal factors in the causation of menstruation to be more involved and less simply told than other sections. This is not, however, the fault of the author as much as of the present unclear status of the problem.

There is properly little mention of clinical hormone therapy, most of it unfortunately in the section dealing with the androgens. The hypophyseal and other gonadotropic hormones are mentioned briefly in several chapters, but enough to indicate their essential importance. The illustrations in this volume, both photographs and line drawings, are excellent. There is less of human reproduction in these chapters than there is of "*mus*, *sus*, and *rhesus*," but it is important that the rôle of these experimental animals in establishing new facts pertaining to human physiology be so fairly interpreted.

It appears to this reviewer that the book will be read by the biologist as an absorbing document of contemporary science. He who is not a biologist has the rare privilege of reading a factual but humanized account of a complex group of functions which loses nothing of validity in being comprehensible.

E. T. Engle

BOOKS RECEIVED

Koch, F. C.: Biological Symposia. Volume IX. Sex Hormones. 10 engravings, 18 text figures 8 tables. Pp. 146. The Jaques Cattell Press.

Krafka, Joseph, Jr.: Human Embryology. 222 illustrations. Pp. 395. Paul B. Hoeber, Inc. $4.75.

Lagerlöf, Henrik O.: Pancreatic Function and Pancreatic Disease. 12 figures, 38 tables. Pp. 289. The Macmillan Company. $3.50.

Snow, William: Clinical Roentgenology of Pregnancy. 119 engravings. Pp. 178. Charles C Thomas.

Werner, August A.: Endocrinology. Clinical Application and Treatment. 2nd edition. 327 engravings and 1 color plate. Pp. 924. Lea and Febiger. $10.00.

Wirtschafter, Zolton Tillson and Morton Korenberg: Diabetes Mellitus. Charts, tables. Pp. 186. Williams and Wilkins.